## CHAPTER I: INTRODUCTION

Attempting to deduce the molecular and cellular basis of certain behaviors in higher organisms such as vertebrates can be a daunting task due to the sheer complexity of neuronal circuits in these organisms. However, when studying an animal with a simpler nervous system, such as the nematode *Caenorhabditis elegans*, elucidation of molecular mechanisms and pathways responsible for behavior is an attainable objective.

Although a relatively simple organism, *C. elegans* is capable of responding to a variety of stimuli including pharmacological treatment, light and heavy touch, temperature, ionic gradients, presence of food and other nematodes. This nematode is an attractive model system for several reasons: (i) it is relatively simple, being composed of approximately a thousand cells, (ii) it contains only three hundred and two neurons which have defined cell lineages and locations, (iii) it has a short life span of approximately four days and is easily maintained in a laboratory environment, being propagated on agar plates with *E. coli* bacteria, (iv) it occurs in two sexes: the self-fertilizing hermaphrodite and males, and (v) it has a small genome (approximately 100 Mbp) which has been sequenced. In addition to these characteristics, *C. elegans* is amendable to classical, molecular and developmental genetic studies, making it particularly useful for defining the role of molecules involved in behavioral processes and the nervous system.

Nicotinic acetylcholine receptors (nAChR's) have been implicated in a variety of *C. elegans* behaviors including feeding (pharyngeal pumping), egg-laying (vulval muscle contraction), locomotion (body muscle contraction) and mating (male spicule protraction) (Kim *et al.* 2001). Though nicotinic receptors are the most thoroughly characterized of ligand-gated ion channels, their function in the molecular mechanisms underlying behavioral processes in even simple model organisms is not well understood.

Briefly, nicotinic receptors are heteropentameric, ligand-gated ion channels with a subunit composition of $\alpha_2 \beta \gamma \delta$. Considerable sequence homology exists between the receptor subunits, which contain four hydrophobic transmembrane helices that produce a functional channel-like structure. When activated by binding of two acetylcholine molecules, nicotinic receptors undergo a conformational change conferring membrane permeability to both sodium and potassium ions, producing an excitatory response lasting only milliseconds. Acetylcholine is bound primarily by the $\alpha$ subunits which contain a pair of cysteine residues although binding also requires residues contributed by the $\gamma$ and $\delta$ subunits.

As previously mentioned, nicotinic receptors function in *C. elegans* egg-laying and locomotion behavior. Egg-laying, which requires a set of eight vulval muscles innervated by cholinergic motorneurons, occurs in a specific temporal pattern involving oscillation between alternate active and inactive phases (Hardaker *et al.* 2001). Periods of activity, in which egg-laying events are clustered together, are separated by a long periods of egg-laying inactivity, as eggs are retained in the uterus (Waggoner *et al.* 1998). Exposure to cholinergic agonists such as nicotine and levamisole has been shown to induce egg-laying, indicating that cholinergic neurotransmission plays a role in the contraction of the vulval muscles involved in egg-laying. In addition, prolonged nicotine treatment has been shown to induce long-term changes in the egg-laying patterns of wild-type animals. Nicotinic receptors also occur in the *C. elegans* body muscle, where they function to mediate muscle contraction involved in locomotion. In fact, the first nicotinic receptor mutants were isolated and identified by their resistance to the stimulatory effects of levamisole on body muscle (Fleming *et al.* 1997).

Several genes encoding homologues of nAChR's have been identified in *C. elegans* yet the roles they play in egg-laying and locomotion behavior remain unresolved. Specifically, we have studied genes of the levamisole receptor, a nicotinic receptor occurring

in both the vulval and body muscles activated by the antihelminthic drug levamisole. Treatment with this agonist causes dose-dependent stimulation of egg-laying and body muscle hypercontraction in wild-type animals (Kim *et al.* 2001). A total of eleven levamisole receptor mutants have been identified which exhibit varying severities of uncoordination and resistance to cholinergic agonists. In addition to retaining a significant portion of normal movement capability, receptor mutants do not exhibit a gross egg-laying defective phenotype (Kim *et al.* 2001).

This thesis documents work aimed at achieving a greater understanding of the role that nicotinic receptor subunit genes play in *C.* elegans egg-laying and locomotion behavior. Briefly, we investigated wild-type and receptor mutant egg-laying response to the cholinergic agonist nicotine, corroborating electrophysiological data indicating the presence of an additional, genetically uncharacterized nicotinic receptor functioning in the body and vulval muscles. Subsequent studies of a nicotine-adaptation defective mutant addressed the regulation of nicotinic receptors. Finally, the role of nicotinic receptors in *C. elegans* locomotion behavior was examined and using an automated behavioral phenotype quantification system.

## CHAPTER II: GENES AFFECTING THE ACTIVITY OF NICOTINIC RECEPTORS INVOLVED IN *CAENORHABDITIS ELEGANS* EGG-LAYING BEHAVIOR


### ABSTRACT

Egg-laying behavior in *C. elegans* is regulated by multiple neurotransmitters, including acetylcholine and serotonin. Agonists of nicotinic acetylcholine receptors such as nicotine and levamisole stimulate egg-laying; however, the genetic and molecular basis for cholinergic neurotransmission in the egg-laying circuitry is not well understood. Here we describe the egg-laying phenotypes of eight levamisole resistance genes, which affect the activity of levamisole-sensitive nicotinic receptors in nematodes. Seven of these genes, including the nicotinic receptor subunit genes *unc-29, unc-38*, and *lev-1*, were essential for the stimulation of egg-laying by levamisole, though they had only subtle effects on egg-laying behavior in the absence of drug. Thus, these genes appear to encode components of a nicotinic receptor that can promote egg-laying but is not necessary for egg-laying muscle contraction. Since the levamisole-receptor mutants responded to other cholinergic drugs, other acetylcholine receptors are likely to function in parallel with the levamisole-sensitive receptors to mediate cholinergic neurotransmission in the egg-laying circuitry. In addition, since expression of functional *unc-29* in muscle cells restored levamisole sensitivity under some but not all conditions, both neuronal and muscle cell UNC-29 receptors are likely to contribute to the regulation of egg-laying behavior. Mutations in one levamisole receptor gene, *unc-38*, also conferred both hypersensitivity and reduced peak response to serotonin; thus nicotinic receptors may play a role in regulating serotonin response pathways in the egg-laying neuromusculature.

## INTRODUCTION

Nicotinic acetylcholine receptors (nAChRs) are heteropentameric ligand-gated ion channels, which induce fast depolarization of excitable cells in response to acetylcholine binding (Galzi *et al*. 1991). In muscle cells, nicotinic receptors present at the neuromuscular junction mediate rapid excitation that leads to muscle contraction. Neurons also contain nicotinic receptors, which are widely expressed in the brain and other neural tissues, and function in the modulation of neurotransmission (Sargent 1995). The activities of nicotinic receptors are known to be subject to both short-term and long-term regulation. For example, long-term exposure to nicotine leads to long-lasting changes in both the abundance and functional activity of nicotinic receptors in brain neurons, processes thought to be critical for nicotine addiction (Dani and Heinemann 1996). Yet the molecular mechanisms responsible for regulating nicotinic receptor activity are not well understood in any organism.

One way to approach the question of nAChR function and regulation in vivo is using a genetically tractable animal such as the nematode *Caenorhabditis elegans*. *C. elegans,* with its simple, well-characterized nervous system and its amenability to classical and molecular genetic studies, is well suited for investigating how specific neurotransmitters, receptors, and signaling molecules function within the context of the nervous system to produce behavior. In *C. elegans*, a number of genes encoding homologues of nAChR subunits have been identified (Ballivet *et al*. 1996; Baylis *et al*. 1997; Fleming *et al*. 1997; Fleming *et al*. 1993; Mongan *et al*. 1998; Treinin and Chalfie 1995). Several of these genes have been shown to encode functional receptor subunits when expressed ectopically in oocytes (Fleming *et al*. 1997; Squire *et al*. 1995); however, the roles most of them play in nervous system function and behavior are not known.

The cells in which nicotinic receptor function has been best characterized in *C. elegans* are the body muscles, which mediate locomotion. Electrical recordings from these muscles indicate that two distinct nicotinic receptor subtypes mediate excitation of the body muscles (Richmond and Jorgensen 1999). The first of these is activated by the antihelminthic drug levamisole, and is therefore known as the levamisole receptor. Activation of this receptor by levamisole causes body muscle hypercontraction and, at high doses, spastic paralysis (Lewis *et al*. 1980b). Screens for levamisole-resistant mutants have led to the identification of multiple genes affecting the function of this receptor (Lewis *et al*. 1980a). Three of these levamisole-resistance genes, *unc-38, unc-29*, and *lev-1*, encode receptor subunits (Fleming *et al*. 1997): *unc-29* and *lev-1* encode candidate non-a subunits of the levamisole receptor, whereas *unc-38* encodes a candidate a subunit. A second, levamisole-insensitive nicotinic receptor has also been identified in the body muscle through electrophysiological methods (Richmond and Jorgensen 1999); the genetics and molecular biology of this receptor have not been characterized.

Nicotinic receptors also function in the pharynx, a specialized muscular organ responsible for feeding. Although the pharynx has intrinsic myogenic contractile activity, cholinergic neurotransmission from the pharyngeal motorneuron MC is necessary for rapid pharyngeal pumping (Raizen *et al*. 1995). Since nicotine but not levamisole induces pharyngeal muscle contraction in the absence of the pharyngeal nervous system, a nicotinic receptor distinct from the levamisole receptor appears to be at least partially responsible for mediating cholinergic transmission in the pharynx (Avery and Horvitz 1990). *eat-2* and *eat-18* are candidates for encoding subunits of this pharyngeal nAChR, since loss-of-function mutations in these genes cause a phenotype similar to ablation of the MC neurons, and in some cases alter pharmacological responses to nicotine (Raizen *et al*. 1995). Another gene,

*deg-3*, encodes a nicotinic receptor subunit that is expressed in the pharynx; however, *deg-3* loss-of-function mutants do not appear to exhibit abnormal feeding behavior (Treinin and Chalfie 1995).

Another *C. elegans* behavior that involves the activity of nicotinic receptors is egg-laying. Egg-laying requires the activity of a set of eight specialized vulval muscles, which are extensively innervated by cholinergic motorneurons (Rand and Nonet 1997a; White *et al*. 1986). Nicotinic agonists including levamisole have been shown to stimulate egg-laying, suggesting that cholinergic neurotransmission involving nicotinic receptors promotes egg-laying muscle contraction (Trent *et al*. 1983; Weinshenker *et al*. 1995). When applied in combination with serotonin, the nicotinic agonist levamisole is capable of stimulating egg-laying muscle contraction in animals carrying ablations of the egg-laying motorneurons (Waggoner *et al*. 1998). This result indicates that stimulation of egg-laying by cholinergic agonists is mediated at least in part by nicotinic receptors in the vulval muscles. *unc-29* recessive mutants are resistant to stimulation of egg-laying by levamisole, and expression of an *unc-29* wild-type transgene in the vulval muscles is sufficient to restore levamisole response (Waggoner *et al*. 2000a). Thus, UNC-29-containing nicotinic receptors in the vulval muscles appear to be at least partially responsible for the acute effects of nicotinic agonists on egg-laying.

In this study, we describe a more detailed analysis of the function and regulation of the nicotinic receptors involved in egg-laying behavior. In particular, we demonstrate that the genes encoding subunits of the well-characterized body muscle levamisole receptor also function in the control of egg-laying, specifically by mediating the stimulation of egg-laying by levamisole, controlling the timing of egg-laying events, and regulating the response of the

egg-laying neuromusculature to serotonin. We also present evidence that the *unc-29* receptor functions in neurons as well as muscle cells.

## RESULTS

### Genes required for egg laying in response to nicotinic agonists

To identify genes required for nAChR function in egg-laying cells, we assayed egg-laying behavior in levamisole-resistant mutants, which were originally identified on the basis of their resistance to the effects of levamisole on body muscle. Mutations conferring resistance to high concentrations of levamisole (i.e., 1 mM) have been identified in several genes, including *unc-29, unc-38, unc-50, unc-63, unc-74*, and *lev-1* (Lewis *et al*. 1980a). All of these "strong" levamisole resistance genes affect the assembly of functional levamisole-binding receptors as assayed in vitro (Lewis *et al*. 1987), and three of them, *unc-38, unc-29*, and *lev-1*, are known to encode nicotinic receptor subunits (Fleming *et al*. 1997). Mutations in three additional genes (*lev-8, lev-9*, and *lev-10*) confer only partial resistance to levamisole (i.e., to concentrations ≤ 100 mM) and have no detectable effect on the biochemical properties of the receptor as assayed in vitro. These "weak" levamisole resistance genes have been hypothesized to regulate the activity of the levamisole receptor indirectly (Lewis *et al*. 1987; Lewis *et al*. 1980a).

To assess the possible involvement of these levamisole resistance genes on egg-laying behavior, we assayed mutant animals for egg-laying in response to acute levamisole treatment. Levamisole treatment results in a dose-dependent stimulation of egg-laying in hypertonic liquid medium (M9 salts), a condition that normally inhibits egg-laying (Trent *et al*. 1983). We had previously found that mutants defective in the nicotinic receptor subunit gene *unc-29* were insensitive to stimulation of egg-laying by levamisole (Waggoner *et al*. 2000a). When we assayed the effects of other levamisole resistance genes, we found that many of them were

also levamisole-resistant with respect to egg-laying (Figure 1). For example, recessive alleles of the *unc-38* and *lev-1* genes, which encode α and non- α nicotinic receptor subunits, respectively, conferred partial or complete resistance to levamisole in the M9 assay. Likewise, mutants defective in the two other "strong" levamisole resistance genes, *unc-63* and *unc-74*, also showed no significant stimulation of egg-laying by levamisole. Finally, *lev-8* and *lev-9* mutants, which were only partially levamisole-resistant in the body muscle, were highly resistant to the stimulatory effects of levamisole on egg-laying. In contrast, *lev-10* mutant animals exhibited a robust stimulation of egg-laying by levamisole. *unc-50* mutants were not tested because their extremely low brood size made egg-laying behavior difficult to evaluate. Together, these results indicated that many of the same genes required for levamisole response in body muscle, including all the known receptor subunit genes, were also required for the acute effects of levamisole on egg-laying. Thus, a nicotinic acetylcholine receptor with a similar subunit composition to the levamisole receptor in body muscle also appeared to promote egg-laying.

To investigate the possible involvement of other acetylcholine receptors in egg-laying behavior, we assayed the egg-laying responses of the levamisole receptor mutants on responses to the general nicotinic agonist nicotine. Like levamisole, nicotine caused robust dose-dependent stimulation of egg-laying by wild-type animals in M9. Mutations in all three known levamisole receptor genes (i.e. *unc-29*, *unc-38* and *lev-1*) led to a reduced response to nicotine in this assay, although significant stimulation was observed in all three mutants, especially at the later time point (Figure 2a, b). Interestingly, the nicotine response observed in the levamisole receptor mutants displayed different dose-response kinetics from the wild-type response; the half-maximal point for the mutant response was at approximately 0.2 mM, compared to 0.8 mM for the wild-type response. Thus, these results indicate that although the

levamisole receptor genes mediate some of the stimulatory effect of nicotine on egg-laying, a significant component of this stimulation is levamisole receptor-independent and may be mediated through a different nicotinic receptor subtype.

**Effects of the levamisole receptor on the temporal pattern of egg-laying**

In order to investigate in more detail the role of nicotinic receptors in egg-laying behavior, we analyzed the effect of the agonist levamisole on the timing of egg-laying events. Under conditions favorable to egg-laying (i.e., nematode growth medium NGM seeded with abundant food), wild-type worms fluctuate between two discrete behavioral states: an inactive egg-laying state during which eggs are retained in the uterus, and an active state during which eggs are laid in clusters. By recording and analyzing an animal's behavior over long time periods, it is possible to determine exponential time constants for the onset of the active phase and for egg-laying within the active phase (Waggoner *et al*. 1998). When we analyzed the egg-laying pattern of wild-type animals during acute levamisole treatment, we observed that the time constant for the onset of the active phase as well as the time constant for egg-laying within the active phase were significantly reduced: on levamisole, the intra-cluster interval displayed a 2-fold reduction, and the inter-cluster interval displayed a more than 3-fold reduction (Figure 3). Thus, levamisole treatment appeared to both facilitate onset of the active phase of egg-laying and stimulate egg-laying muscle contractions during the active phase. These effects of levamisole on the timing of egg-laying events were dependent on the levamisole receptor genes (*unc-29*, *unc-38* and *lev-1*). By themselves, these genes had only subtle effects on the temporal pattern of egg-laying (Table 1). Yet all mutants retained the characteristic biphasic pattern of egg-laying, and the mutant time constants differed significantly from those of wild-type by no more than a factor of two. Yet in contrast to its effect on wild-type animals, levamisole treatment had little or no effect on the egg-laying

patterns of the levamisole receptor mutants (Figure 3; *unc-38* and *lev-1* data not shown). Thus, although the levamisole receptor genes were not critical for egg-laying in the absence of drug, they were apparently necessary for the acute effects of levamisole on egg-laying on NGM as well as in M9.

How likely is it that these mutations in the nicotinic receptor genes completely eliminate the functional activity of the encoded receptor protein? The previously reported sequences of the *unc-38(x20)* and *lev-1(x427)* alleles suggest that they cause severe if not complete loss of receptor function (Fleming *et al*. 1997). By sequencing the complete coding regions of the *unc-29* mutant alleles, we determined that the *unc-29(x29)* mutation introduced a nonsense mutation (tat → taa) within the fourth transmembrane alpha-helix. *unc-29(e193)* was found to be a missense mutation (cca → tca) that changes a universally conserved proline residue at position 258 to a serine; the sequence alteration in the *unc-29(e1072)* allele could not be identified. Thus, *unc-29(x29)* probably causes a severe loss of nicotinic receptor function and might represent a molecular null allele. To further address the egg-laying phenotype of a complete levamisole receptor knockout, we analyzed the egg-laying behavior of *unc-29/unc-38/lev-1* double and triple mutant strains, which were defective in two (or in the latter case three) of the identified levamisole receptor subunits. In each case, the pattern of egg-laying was grossly normal, and although abnormalities in the timing of egg-laying events were observed, they were quantitatively not much more severe than in the single mutant strains (Figure 3; Table 1). Together, these results suggested that while a nicotinic receptor containing UNC-29, UNC-38, and LEV-1 subunits is necessary for stimulation of egg-laying by levamisole, it is unnecessary for egg-laying muscle contraction per se.

**The cellular basis for *unc-29* egg-laying phenotypes**

UNC-29 is expressed in both neurons and muscle cells (Fleming *et al*. 1997). Specifically, UNC-29 has been shown to be expressed in the vulval muscles as well as the VC egg-laying motorneurons ((Waggoner *et al*. 2000a); see Figure 4a). Thus, it was of interest to determine the cellular foci for the egg-laying phenotypes of *unc-29*. In principle, the egg-laying abnormalities in *unc-29* loss-of-function mutants could be the result of a lack of UNC-29 receptor function in the vulval muscles, the VCs, or perhaps some other cell type. To address these possibilities, we generated transgenic animals with the *unc-29* mutant background that expressed the wild-type allele of *unc-29* only in muscle cells (using the *myo-3* promoter (Okkema *et al*. 1993)) or only in the vulval muscles (using the *ndE-box* promoter (Harfe and Fire 1998)). When we tested these lines for the ability to lay eggs in M9 in response to levamisole, we observed that both the *myo-3::unc-29* line and the *ndE-box::unc-29* line showed robust stimulation (Figure 4b). Thus, functional UNC-29 receptors in the vulval muscles apparently were sufficient to rescue the levamisole-insensitive phenotype as measured in the M9 assay.

We also assayed the responses of our muscle-specific transgenic lines to levamisole on NGM agar plates. Surprisingly, the egg-laying patterns of both the *myo-3::unc-29* line (Figure 4c) and the *ndE-box::unc-29* line (not shown) were largely unaffected by the presence of levamisole. Thus, whereas vulval muscle-specific expression of wild-type *unc-29* rescued the levamisole-resistant phenotype of *unc-29(x29)* in the M9 egg-laying assay, muscle-specific expression failed to rescue the levamisole resistant phenotype on NGM plates. These results implied that vulval muscle UNC-29 receptors were sufficient to provide levamisole response in M9, whereas levamisole response on NGM might require the activity of neuronal UNC-29 receptors. To test this possibility, we performed ablations that eliminated the egg-laying

motorneurons that express UNC-29 (i.e. the VCs), and determined the effect of this ablation on egg-laying responses to levamisole. We observed that like the *myo-3::unc-29* animals, animals lacking the VCs were largely levamisole-insensitive on NGM (Figure 4d). These results were consistent with the hypothesis that levamisole receptors in the VC neurons participate in the control of egg-laying.

**Effect of levamisole receptor genes on serotonin responses**

Previous studies indicated that acetylcholine and serotonin act in parallel to activate egg-laying in *C. elegans*. To gain further information about the relationship between serotonin and acetylcholine in the control of egg-laying, we analyzed the behavior of double mutants defective in both nicotinic acetylcholine receptor function and serotonergic neurotransmission. We first analyzed the phenotypes of double mutants carrying a loss of function mutation in *tph-1*, which encodes the serotonin biosynthetic enzyme tryptophan hydroxylase (Sze *et al*. 2000). We observed that by itself, a *tph-1* loss-of-function mutation resulted in a small but significant increase in the duration of the inactive egg-laying phase. When an *unc-29* or *lev-1* mutation was crossed into a *tph-1* mutant background, no significant enhancement of the egg-laying phenotype was observed (Figure 5a-c). In contrast, both *unc-29* and *lev-1* dramatically enhanced the egg-laying defect caused by an *egl-1* mutation, which causes inappropriate cell death of the serotonergic HSN neurons (Desai *et al*. 1988). In an *egl-1* mutant background, *unc-29* and *lev-1* mutations decreased the overall rate of egg-laying and significantly decreased the rate of egg-laying within the active phase (Figure 5d-f). Thus, the activity of UNC-29/LEV-1 containing nicotinic receptors appeared to be particularly important for egg-laying in the absence of the HSNs.

We also assayed the effect of mutations in the levamisole receptor genes on the egg-laying response to exogenous serotonin (Figure 6). We observed that *unc-38* mutants

exhibited a greatly overall diminished response to serotonin; at peak concentrations, *unc-38* mutants showed at least a three-fold reduction in the number of eggs laid (Figure 6c). In addition, *unc-38* mutants also showed hypersensitivity to serotonin, in that they consistently responded more than wild-type to abnormally low concentrations of drug. Since *unc-38* mutations also conferred serotonin hypersensitivity in an *egl-1* mutant background (data not shown), this effect was apparently independent of the HSN neurons. Thus, *unc-38* appeared to both potentiate and negatively regulate serotonin response in the egg-laying circuitry. In contrast, the dose responses of *unc-29* and *lev*-1 mutants were more similar to wild-type: although both mutants did experience a reduction in peak response, it was not nearly as pronounced as that seen in *unc-38* mutants, and statistically significant serotonin hypersensitivity to low doses was seen in only one allele of *unc-29* (*(e193;* Figure 6a, b).

## DISCUSSION

### Genetic requirements for cholinergic neurotransmission in egg-laying behavior

Cholinergic agonists, including the nicotinic agonists nicotine and levamisole, have long been known to stimulate egg-laying. We recently found that the stimulation of egg-laying by levamisole requires a nicotinic receptor containing the subunit protein UNC-29, which is also an essential component of the levamisole receptor in body muscle (Waggoner *et al*. 2000a). In this study we observed that several other genes that affect the activity of the body muscle levamisole receptor are also required for the acute effects of levamisole on egg-laying. These include the genes *unc-38* and *lev-1*, which encode a and non- a subunits, respectively, of the body muscle levamisole receptor (Fleming *et al*. 1997). Thus, the levamisole-sensitive nicotinic receptor that promotes egg-laying muscle contraction appears to have a similar if not identical subunit composition to the levamisole receptor in the body muscle. Interestingly, *lev-1* loss-of-function mutants, which are only partially resistant to

levamisole with respect to body muscle contraction, were completely levamisole-resistant with respect to egg-laying. Therefore, the LEV-1 protein may be a nonessential subunit of the body muscle levamisole receptor, but an essential subunit of the levamisole receptor involved in egg-laying. Several other levamisole resistance genes that have not been cloned, including *unc-74*, *lev-8*, and *lev-9*, also appeared to affect egg-laying in response to nicotinic agonists (though only a single allele of *lev-8* was available, making effects of genetic background impossible to rule out for this mutant). Thus, the regulatory pathways controlling the activity of levamisole receptors involved in egg-laying may be also similar to those affecting the body muscle receptors.

Despite their apparent role in mediating cholinergic neurotransmission in the egg-laying circuit, none of the levamisole resistance genes were critical for egg-laying. Recessive mutants with defects in the levamisole receptor subunit genes not only failed to exhibit a gross egg-laying defective phenotype, but their temporal patterns of egg-laying also showed little deviation from those of wild-type animals. Even double and triple mutants defective in multiple levamisole receptor subunits exhibited only a subtle alteration in egg-laying pattern: the intra-cluster and inter-cluster time constants were slower by a factor of no more than 2. *unc-29*, *unc-38*, and *lev-1* mutants all exhibited a reduced but measurable response to the general nicotinic agonist nicotine; thus, the vulval muscles, like the body muscles, most likely contain a second nicotinic receptor whose function overlaps that of the levamisole receptor (Richmond and Jorgensen 1999). It is also possible that the vulval muscles, like the pharyngeal muscles, may possess intrinsic myogenic activity, making cholinergic neurotransmission important but not essential for muscle contraction (Raizen *et al*. 1995). The egg-laying phenotypes of choline acetyltransferase (i.e., *cha-1*) mutants support this possibility; *cha-1* conditional mutants have a significantly longer intra-cluster time constant

than even the levamisole receptor triple mutant (Waggoner *et al*. 1998), yet not even the strongest *cha-1* mutants are completely defective for egg-laying (Rand 1989). In the future, analyses of vulval muscle physiology may make it possible to address this issue more definitively.

**Evidence that *unc-29* functions in both egg-laying muscles and neurons**

Although the function of UNC-29-containing nicotinic receptors has previously been studied only in muscle cells, GFP reporter studies have indicated that UNC-29 receptors are also expressed in neurons (Fleming *et al*. 1997). The evidence presented here indicates that neuronal as well as vulval muscle UNC-29 receptors participate in the control of egg-laying behavior. Expression of a functional *unc-29* transgene under the control of the vulval muscle-specific *ndE-box* promoter restored levamisole sensitivity in the M9 egg-laying assay; thus, it is clear that UNC-29 receptors in the vulval muscles mediate at least some of the effects of nicotinic agonists on egg-laying, probably through direct muscle excitation. However, when analyzed on NGM plates, lines expressing *unc-29(+)* under the control of either the *ndE-box* or the muscle-specific *myo-3* promoter appeared levamisole-resistant, since their egg-laying pattern was largely unaffected by levamisole treatment. These results illustrate that the genetic requirements for pharmacological stimulation of egg-laying can differ when assayed under conditions that are normally permissive for egg-laying (i.e. seeded NGM plates) instead of conditions that are normally inhibitory (i.e. M9 liquid medium). Such a discrepancy is not unique to these transgenic lines; we previously observed that mutants defective in the neuropeptide gene *flp-1* showed almost no stimulation of egg-laying by serotonin in M9, but displayed an essentially wild-type response on NGM (Waggoner *et al*. 2000b). Since the *myo-3* promoter directs gene expression in all *unc-29*-expressing muscle cells at levels at least as high as those provided by the *unc-29* promoter (see (Waggoner *et al*. 2000a)), our results

suggest that neuronal UNC-29 receptors are important for the levamisole response on NGM. Consistent with this possibility, ablations that eliminated the VC motorneurons (the only neurons in the egg-laying circuit with detectable UNC-29 expression) also conferred resistance to levamisole in the NGM assay. Since an ectopic promoter that replicated the neuronal UNC-29 expression pattern was not available, we could not determine whether neuronal UNC-29 receptors were sufficient for levamisole response on NGM. Nonetheless, our results indicate that the alterations in egg-laying pattern caused by levamisole treatment are at least partially dependent on neuronal UNC-29 receptors, possibly functioning in the VCs.

How might neuronal UNC-29 receptors participate in the regulation of egg-laying? In vertebrate systems, neuronal nicotinic receptors have been shown to facilitate neurotransmitter release from synaptic terminals. Thus, it is reasonable to suppose that UNC-29 receptors in the VCs function in a similar fashion to promote release of neurotransmitters and/or neuromodulators that function at the VC-vulval muscle neuromuscular junctions. The VCs contain multiple neurotransmitters, including acetylcholine (Rand and Nonet 1997a), one or more FMRFamide-related neuropeptides (Schinkmann and Li 1992), and an unidentified biogenic amine (Duerr *et al*. 1999). Since levamisole shortens, and levamisole receptor mutations often lengthen, both the inter-cluster and intra-cluster time constants, levamisole receptors in the VCs may regulate the release of transmitters that induce egg-laying contractions within the active phase (i.e. acetylcholine) as well as modulators that control the onset of the active phase (perhaps a peptide or amine).

**Levamisole receptors and HSN-independent egg-laying neurotransmission**

Although mutations in the levamisole receptor genes had relatively subtle effects on the pattern of egg-laying, their effect was considerably greater in animals lacking the

serotonergic HSN motorneurons. In an *egl-1* mutant background, both *unc-29* and *lev-1* mutations lengthened the intra-cluster time constant more than three-fold. These results imply that the levamisole receptor is specifically important for cholinergic neurotransmission between the VC motorneurons and the vulval muscles, but less important for HSN/vulval muscle neurotransmission. Neuronal UNC-29 receptors indeed appear to be expressed in the VCs but not the HSNs; thus, importance of *unc-29* for HSN-independent egg-laying may reflect this asymmetry in the distribution of neuronal UNC-29 receptors in the egg-laying motor synapses.

Interestingly, no phenotypic synergy was observed between the levamisole receptor genes and *tph-1*, a gene required for the synthesis of the major HSN neurotransmitter serotonin. This is perhaps surprising since serotonin is sufficient to rescue HSN function (Trent *et al*. 1983) and has been shown to potentiate the induction of egg-laying by nicotinic agonists (Waggoner *et al*. 1998). However, a number of studies have demonstrated that the loss of HSN function can have effects on egg-laying that are more severe than those caused by a defect in serotonergic neurotransmission. For example, HSN-deficient animals were found to be resistant to stimulation of egg-laying by levamisole, whereas serotonin-deficient animals were not (Weinshenker *et al*. 1995). Likewise, the ability of $G_o$/*goa-1* mutations to shorten the inactive egg-laying phase is dependent on the presence of the HSNs, but not on the ability to synthesize serotonin (Waggoner *et al*. 2000b). The simplest interpretation of these results is that the HSNs contain a second neuromodulator that functions redundantly with serotonin to potentiate the ability of nicotinic receptors to induce vulval muscle contraction. The identity of such a hypothetical stimulatory modulator of egg-laying is not known, though since the HSNs contain RFamide immunoreactivity, one possible candidate is a FMRFamide-related neuropeptide.

**Connections between nicotinic receptor activity and serotonin response**

An interesting and unexpected result was the observation that mutants defective in the receptor subunit gene *unc-38* dramatically altered the egg-laying response to serotonin. *unc-38* animals laid eggs in response to serotonin concentrations that were approximately five-fold lower than those required to stimulate egg-laying in wild-type; however, the magnitude of the serotonin response (i.e. the maximum number of eggs laid in response to serotonin) was much lower in *unc-38* mutants than in wild-type animals. Both the hypersensitivity and reduced response to serotonin appeared to be somewhat specific to *unc-38*, as both these effects were minimal in *unc-29* and *lev-1*; overall, the serotonin dose responses of these two mutants were more similar to wild-type. Thus, a nicotinic receptor containing UNC-38 but not UNC-29 or LEV-1 subunits appears to both promote and negatively regulate serotonin responses in the egg-laying circuitry.

What interactions between cholinergic and serotonergic response pathways might explain the effects of *unc-38* mutations on serotonin response? Interestingly, mutations in a number of genes involved in promoting neurotransmitter release from ventral cord neurons are also serotonin-hypersensitive and/or show reduced serotonin responses (Schafer *et al*. 1996). Preliminary analysis of *unc-38::GFP* promoter fusions (A. Gottschalk and W. Schafer, unpublished) as well as genetic evidence (discussed in (Rand and Nonet 1997b)) suggests that *unc-38* functions in neurons as well as muscles. Perhaps neuronal UNC-38 receptors might control the release of modulators that downregulate or potentiate serotonin response in the egg-laying neuromusculature. Alternatively, the vulval muscles might express an UNC-38-containing nicotinic receptor subtype whose chronic activity regulates the activity of signal transduction pathways downstream of serotonin. In either case, the long-term activity of UNC-38 receptors could directly or indirectly modulate the activity of serotonin-responsive

signaling pathways in the vulval muscles. In the future, the identification and characterization of additional genes required for the egg-laying responses to these transmitters are likely to provide insight into these interactions between cholinergic and serotonergic neurotransmission.

## METHODS

### Strains and Genetic Methods

The chromosomal locations of the genes studied in these experiments are as follows: LGI: *unc-74, unc-38, unc-63, unc-29, lev-10*; LGII: *tph-1*; LGIV: *dpy-20, lev-1*; LGV: *egl-1*; LGX: *lev-9, lev-8*. Routine culturing of *C. elegans* was performed as described (Brenner 1974). All mutant strains but one, (*lev-9(x66)*), had been backcrossed once to wild-type when we received them; for the tracking experiments (Table 1), the *unc-29* and *lev-1* mutant strains were each backcrossed an additional four times to wild-type.

### Behavioral Assays

Drug response assays in M9 salts were performed essentially as described (Waggoner *et al*. 2000a). Unless otherwise stated, nematodes were grown at room temperature on standard nematode growth medium (NGM) seeded with *E. coli* strain OP50 as a food source. For dose response experiments, individual young, gravid hermaphrodites were placed in microtiter wells containing liquid M9 and the indicated concentration of drug. After a 1 hour incubation at room temperature, the eggs laid by each animal were counted. For studies of egg-laying behavior on NGM solid media, single animals were transferred to agar plates seeded with *E. coli* OP50 as a food source. The egg-laying behavior of each animal was recorded for 4-12 hours as described using an automated tracking system (Waggoner *et al*.

1998). Because levamisole treatment emptied the uterus of wild-type animals, wild-type worms were tracked on levamisole for only 2 hours.

**Analysis of Egg-Laying Patterns**

Intervals between egg-laying events were determined from analysis of videotapes obtained using the tracking system. Quantitative analysis of the egg-laying pattern using this interval data was performed as described (Zhou *et al*. 1998). Briefly, egg-laying events in *C. elegans* are clustered, with periods of active egg-laying, or active phases, separated by long inactive phases during which eggs are retained. Both the duration of the inactive phases ("inter-cluster intervals") and the duration of intervals between egg-laying events in a cluster ("intra-cluster intervals") model as exponential random variables with different time constants (Waggoner *et al*. 1998). Thus, the probability density function for the intervals between events is

$$f_X(x) = k_1\ \lambda_1\ e^{-\lambda_1 x} + k_2\ (p\lambda_2)\ e^{-(p\lambda_2)x}, \qquad x \geq 0,$$

$$k_1 = \frac{p(\lambda_1 - \lambda_2)}{\lambda_1 - p\lambda_2}, \qquad k_2 = \frac{\lambda_1(1-p)}{\lambda_1 - p\lambda_2}.$$

, where the intra-cluster time constant is $1/l_1$ and the inter-cluster time constant is $1/pl_2$. The maximum likelihood estimation method used to derive timing parameters was essentially the one described previously (Zhou *et al*. 1998), with one improvement: multiple histograms of log interval time were used to initialize the ML algorithm, thereby avoiding the possibility of the program fixing on a local rather than the global maximum.

The experimental variance and statistical significance of the timing data were evaluated in two ways. The theoretical expected variance of estimated parameters and time constants based on the two-state model was determined by using the model probability density function to generate 100 independent sets of simulated egg-laying data (containing the same

number of intervals as the real data and using the same parameters) , and computing the standard deviation of the parameters estimated from these simulations. Analysis of real data using this method indicates that real variation between individual animals of the same strain is comparable to the theoretical expectation. For example, when 8 recordings of different wild-type worms made on different days were individually analyzed, the average intra- and inter-cluster time constants estimated from each were 14 s and 1374 s, with standard deviations of 7 s and 422 s, respectively. Simulations using the same parameters and data sets of comparable size (approximately 40 intervals) had similar variation to what was observed experimentally (standard deviation: 4s and 504s). To test for the statistical significance of differences in egg-laying interval times, a non-parametric test was used (the Mann-Whitney rank test). The ability of such tests to determine statistical significance is independent of the nature and degree of variation in the data (Zar 1996). To increase our confidence that differences between mutant and wild-type strains were due to mutations in the levamisole receptor genes, all *unc-29* and *lev-1* mutant strains were backcrossed four times to wild-type (in addition the one backcross performed prior to our receiving the strains) before being analyzed in the tracking assay.

**Sequencing of *unc-29* mutations**

Genomic DNA (Sulston and Hodgkin 1988) or single worms (Williams 1995) were used as a template to obtain PCR products for sequencing. PCR products were purified using the QIAquick Gel Extraction Kit. DNA sequencing was performed by the Molecular Pathology Shared Resource, UCSD Cancer Center, which is funded in part by NCI Cancer Center Support Grant #5P0CA23100-16. The sequence alterations were confirmed by both sequencing DNA from additional independent PCR reactions and by restriction digest of multiple PCR products from single worms.

**Construction of double and triple mutant strains**

Double mutants carrying mutations in *tph-1* or *egl-1* and in one of the levamisole receptor genes were constructed crossing the single mutants and screening the second generation self-progeny for nicotine-resistant animals whose progeny were all egg-laying defective. The *unc-38(sy576) unc-29(e1072)* double mutant was derived by crossing a *unc-38(sy576) unc-29(e1072); him-5(e1490)* strain (provided by Rene Garcia and Paul Sternberg) to wild-type, and isolating an Unc non-Him F2 segregant . *lev-1(e211)* was introduced into this double mutant or into *unc-38(x20)* or *unc-29(x29)* in the following manner. *lev-1(e211)* males were mated to the Unc strain and the nicotine-sensitive hermaphrodite progeny were then mated to *lev-1(e211)* males. Non-Unc nicotine-resistant (i.e. *lev-1/lev-1*) hermaphrodite progeny from this backcross were picked individually and allowed to self-fertilize. Some of these segregated Unc animals, which were picked individually and allowed to self-fertilize. The presence of *unc-38*, *unc-29*, and *lev-1* was confirmed by test cross with males heterozygous for *unc-38(x20)* or *unc-29(x29)* or males homozygous for *lev-1(e211)*.

**Cell Ablation Experiments**

For ablations of VC1-6, we ablated the neuroblasts P1.a-P9.a, which are the larval precursors of the VCs. Although only P3.a-P8.a normally give rise to the VC's, adjacent Pn.a cells can generate VC's in the absence of P3.a-P8.a unless killed (Li and Chalfie 1990). Wild-type animals were grown at 20$^{\infty}$ C; approximately 10 hours after hatching, the Pn.a cell nuclei were identified by position in the ventral cord and killed; cell killing was verified by scoring for the absence of adult ventral cord motorneuronal nuclei in the mid-body region. We also confirmed the ability of this ablation to eliminate the VCs by performing the same procedure on strain AQ242 (genotype: *dpy-20(e1282)IV; ljEx1[dpy-20(+), egl-36::GFP]*), which expressed GFP in all VC neurons; cell killing was verified in late L4 by scoring for the

absence of fluorescence in the ventral cord and vulval area. Three other ventral cord neurons which also descend from the ablated neuroblasts (VA7, VB8, and VD7) potentially make single synapses with the egg-laying muscles; thus, it was formally possible these neurons might contribute to this effect. However, ablations of a subset of Pn.a neuroblasts (for example, P3.a-P.8.a) which eliminated VA7, VB8, and VD7 but spared one or more VCs (as indicated by *egl-36::GFP* expressing cells that sent processes to the vulva) did not prevent levamisole response (data not shown).

This chapter is, in full, a reprint of material as it appears in J. Kim et al. (2001) "*Genes Affecting the Activity of Nicotinic Receptors Involved in Caenorhabditis elegans Egg-Laying Behavior*" (Genetics 157: 1599–1610 (April 2001)). The thesis author was a secondary researcher, responsible for all data appearing in Figure 2.

**Table 1: Egg-laying patterns of levamisole-receptor mutants**

| Strain (#, hrs, intervals) | Intra-cluster time constant ($1/\lambda_1$; s) | Inter-cluster time constant ($1/p\lambda_2$; s) | Clustering parameter (p) |
|---|---|---|---|
| N2 (10, 58, 357) | 16 ± 2 | 1386 ± 201 | 0.555 ± 0.031 |
| *unc-29(x29)* (8, 38, 196) | 14 ± 2 | 2236* ± 494 | 0.700 ± 0.057 |
| *unc-29 (e193)* (8, 44, 155) | 16 ± 4 | 2042* ± 267 | 0.327 ± 0.051 |
| *unc-29 (e1072)* (8, 44, 162) | 31* ± 6 | 1733* ± 236 | 0.444 ± 0.045 |
| *unc-38 (x20)* (6, 25, 127) | 18 ± 3 | 1738 ± 389 | 0.669 ± 0.043 |
| *unc-38 (e264)* (15, 62, 207) | 18 ± 4 | 1555 ± 166 | 0.357 ± 0.035 |
| *lev-1 (e211)* (10, 45, 161) | 32* ± 5 | 2171* ± 296 | 0.497 ± 0.040 |
| *lev-1 (x427)* (11, 52, 149) | 28* ± 4 | 2896* ± 477 | 0.508 ± 0.048 |
| *unc-29(x29); lev-1(e211)* (7, 31, 43) | 30 ± 5 | 2067 ± 417 | 0.576 ± 0.047 |
| *unc-38(x20); lev-1(e211)* (12, 40, 138) | 27* ± 4 | 2007* ± 305 | 0.453 ± 0.048 |
| *unc-38(sy576); unc-29(e1072)* (7, 27, 113) | 29* ± 5 | 1740* ± 324 | 0.479 ± 0.053 |
| *unc-38(sy576); unc-29(e1072); lev-1(e211)* (8, 30, 118) | 32* ± 7 | 1850* ± 330 | 0.492 ± 0.050 |

* short(<120s) or long (>120s) intervals statistically different (level of confidence $p < .05$) from wild-type according to the Mann-Whitney rank sum test.

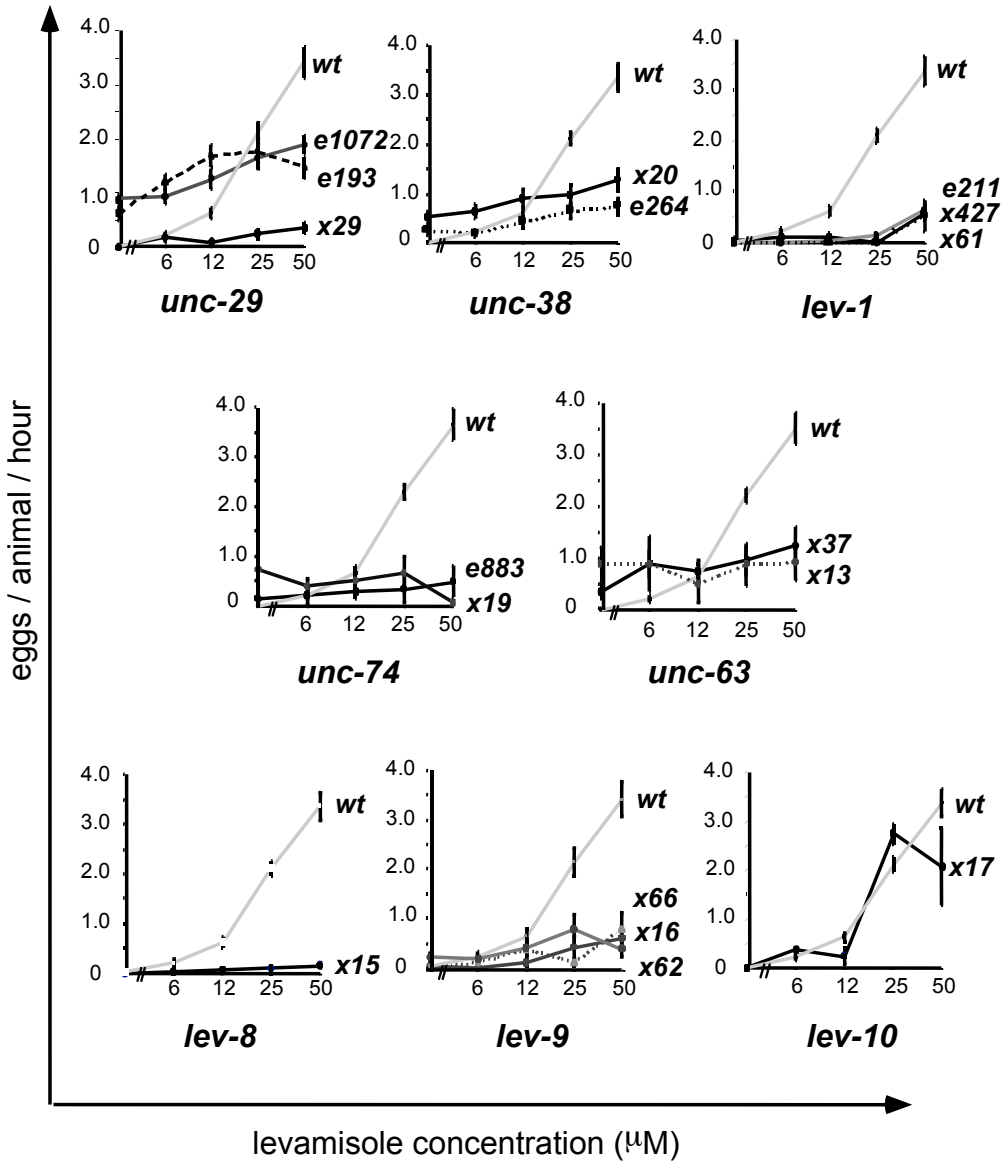



**Figure 1: Effects of levamisole resistance genes on egg-laying in response to levamisole.** Egg-laying responses to levamisole were determined for the different mutant alleles by placing individual animals in liquid M9 and the indicated concentration of levamisole, and counting the number of eggs laid by each animal after 1 hour of drug exposure. *unc-29* data (adapted from (Waggoner *et al*. 2000a)) are shown for comparison. Animals carrying mutations in *unc-38, unc-63, unc-74, lev-1, lev-8*, and *lev-9* showed a statistically significant reduction in levamisole-induced egg-laying according to the Mann-Whitney rank sum test (level of confidence $p < .001$). Individual points and error bars indicate the mean and SEM of the following numbers of trials: N2=197, *unc-29(e193)*=144, *unc-29(e1072)*=96, *unc-29(x29)*=96, *unc-38(x20)*=96, *unc-38(e264)*=96, *lev-1(e211)*=96, *lev-1(x427)*=96, *lev-1(e61)*=48, *unc-74(e883)*=38, *unc-74(x19)*=26, *unc-63(x37)*=40, *unc-63(x13)*=28, *lev-8(x15)*=72, *lev-9(x16)*=72, *lev-9(x62)*=30, *lev-9(x66)*=30, *lev-10(x17)*=96.

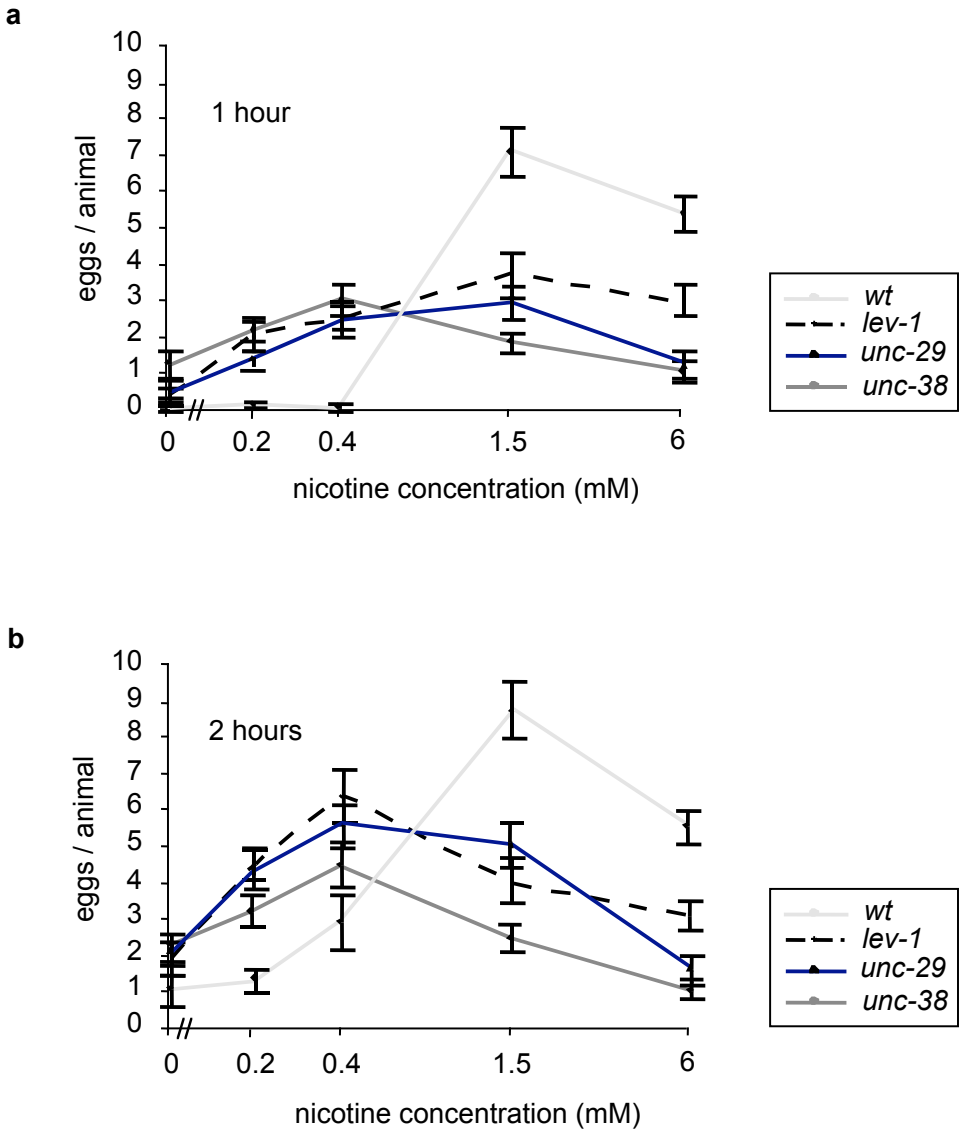



**Figure 2: Effects of levamisole receptor genes on egg-laying in response to the general nicotinic agonist nicotine.** Egg-laying responses to nicotine were determined using the M9 assay described in Figure 1. Data collected after 1 and 2 hours are shown in parts a and b, respectively. The *unc-29(x29), lev-1(e211)* and *unc-38(x20)* alleles were used for these experiments. Points and error bars indicate the mean and standard error of 36 trials at each concentration.

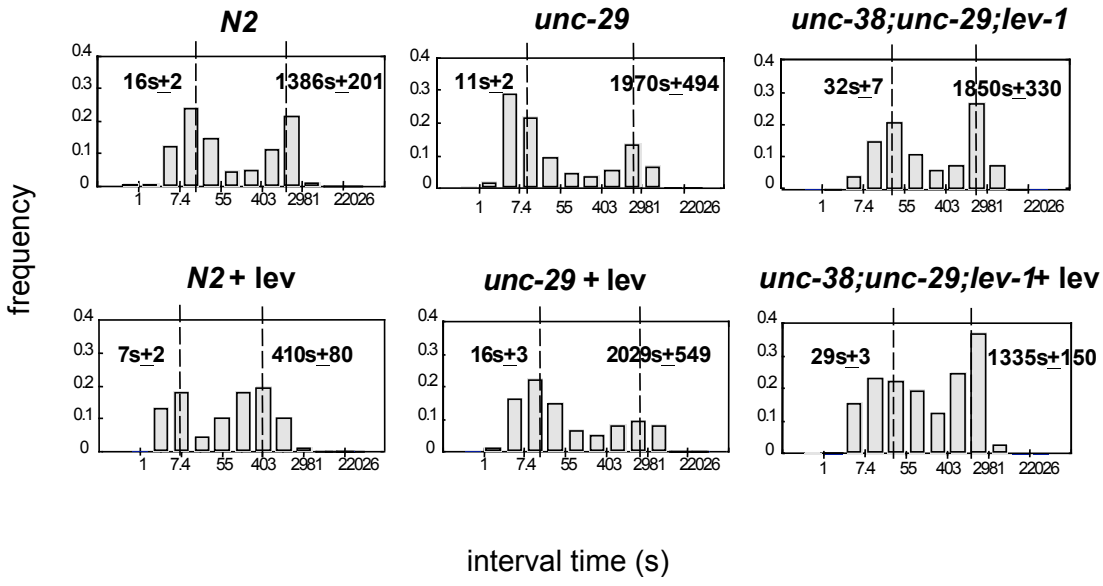



**Figure 3: Effect of the levamisole receptor on the timing of egg-laying events.** Shown are histograms of interval times between egg-laying events for wild-type or mutant animals in the presence or absence of levamisole. For each histogram, the left peak contains the intra-cluster intervals (i.e., the intervals between events within a cluster), and the right peak contains the inter-cluster intervals (i.e., the intervals between clusters). Dashed lines indicate the estimated intra-cluster and inter-cluster time constants. Animals were tracked as described on nematode growth medium (NGM) or on NGM containing 50 μM levamisole. This concentration effectively stimulates egg-laying in wild-type animals, but does not cause body muscle paralysis. For animals tracked on NGM, the numbers of animals tracked, hours observed, and intervals analyzed, along with the estimated model egg-laying parameters, are in Table 1. For animals tracked on levamisole, the numbers of animals, hours tracked, and total intervals analyzed were: N2--9 animals, 19 hrs, 64 intervals; *unc-29(x29)*--5 animals, 21 hrs, 100 intervals; *lev-1(e211)*--6 animals, 28 hrs, 101 intervals; *unc-38(x20)*--7 animals, 35 hrs, 104 intervals; *unc-38(sy576) unc-29(e1072); lev-1(e211)*--9 animals, 47 hrs, 275 intervals. Both the long (>120s) and short (<120s) intervals were significantly shortened by levamisole in wild-type (level of confidence $p<.001$). In the *unc-29* single mutant neither parameter was significantly shortened, and in the *unc-38;unc-29;lev-1* triple mutant the long interval was slightly shortened (level of confidence $p<.05$) according to the Mann-Whitney rank sum test. In testing the significance for the mutants, a cut-off point of 300s was used in separating long and short intervals to adapt to the shifts in the curves representing intra-cluster intervals.

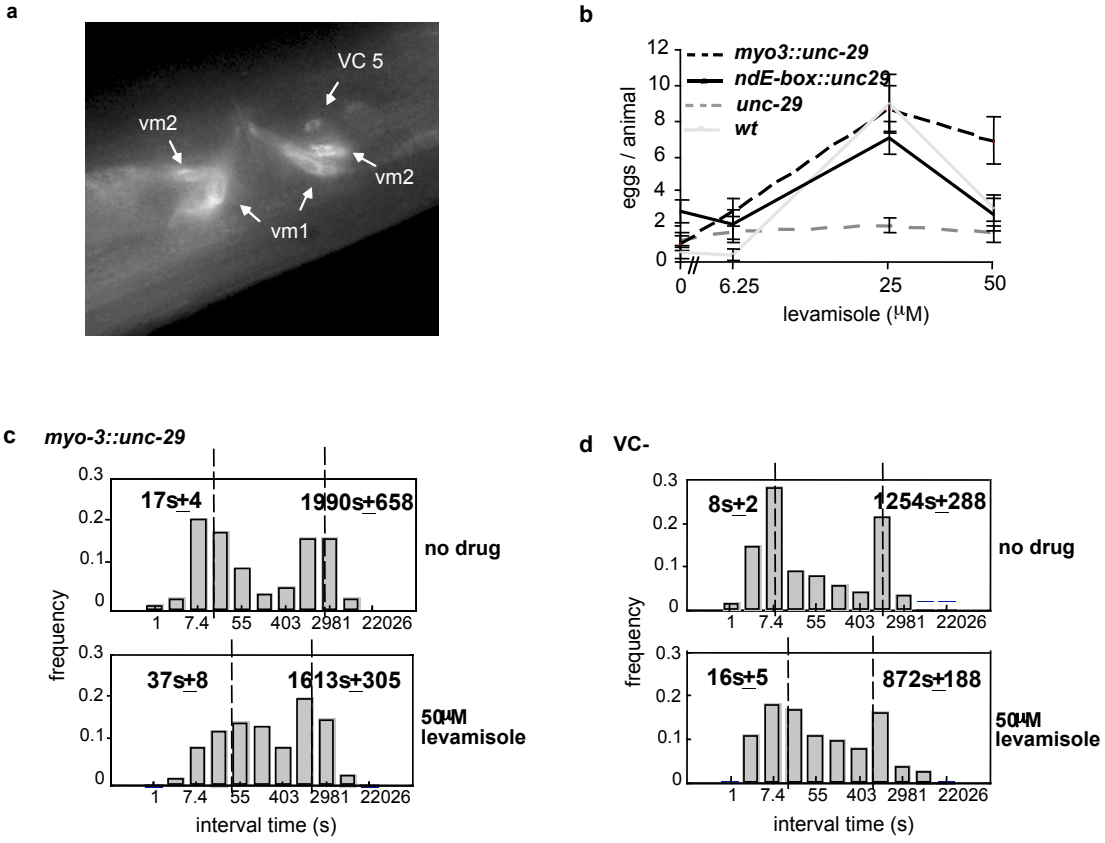



**Figure 4: Partial rescue of *unc-29* egg-laying phenotypes by muscle-specific gene expression.** a. Expression pattern of UNC-29 in the egg-laying neuromusculature. An UNC-29::GFP chimeric protein shows pattern of expression in the vulval muscles (vm1 and vm2) and VC motorneurons (Waggoner *et al*. 2000a). b. Dose response curves showing egg-laying in response to levamisole by wild-type animals, *unc-29* mutants, and transgenic animals expressing functional *unc-29* only in the vulval muscles (*ndE-box::unc-29(+)*; strain AQ497) or in vulval and body muscles (*myo-3::unc-29*; strain AQ548) in an *unc-29* mutant background. Individual points and error bars indicate the mean and SEM of at least the following numbers of trials: N2=16, *unc-29(x29)=16, ndE-box::unc-29(+)*=20, *myo-3::unc-29*(+)=30. c-d. Effect of levamisole on the egg-laying patterns of animals with muscle specific *unc-29(+)* expression (c) or with ablation of the Pn.a VC neuronal precursors (d). Dashed lines indicate the estimated intra-cluster and inter-cluster time constants. The number of animals, hours tracked, and total intervals analyzed were: AQ548/*myo-3::unc-29* (no drug): 5 animals, 26 hr, 71 intervals; AQ548/*myo-3::unc-29* (levamisole): 5 animals, 30 hr, 109 intervals; N2, VC- (no drug): 3 animals, 20 hr, 42 intervals; N2, VC- (levamisole): 3 animals, 24 hr, 69 intervals.

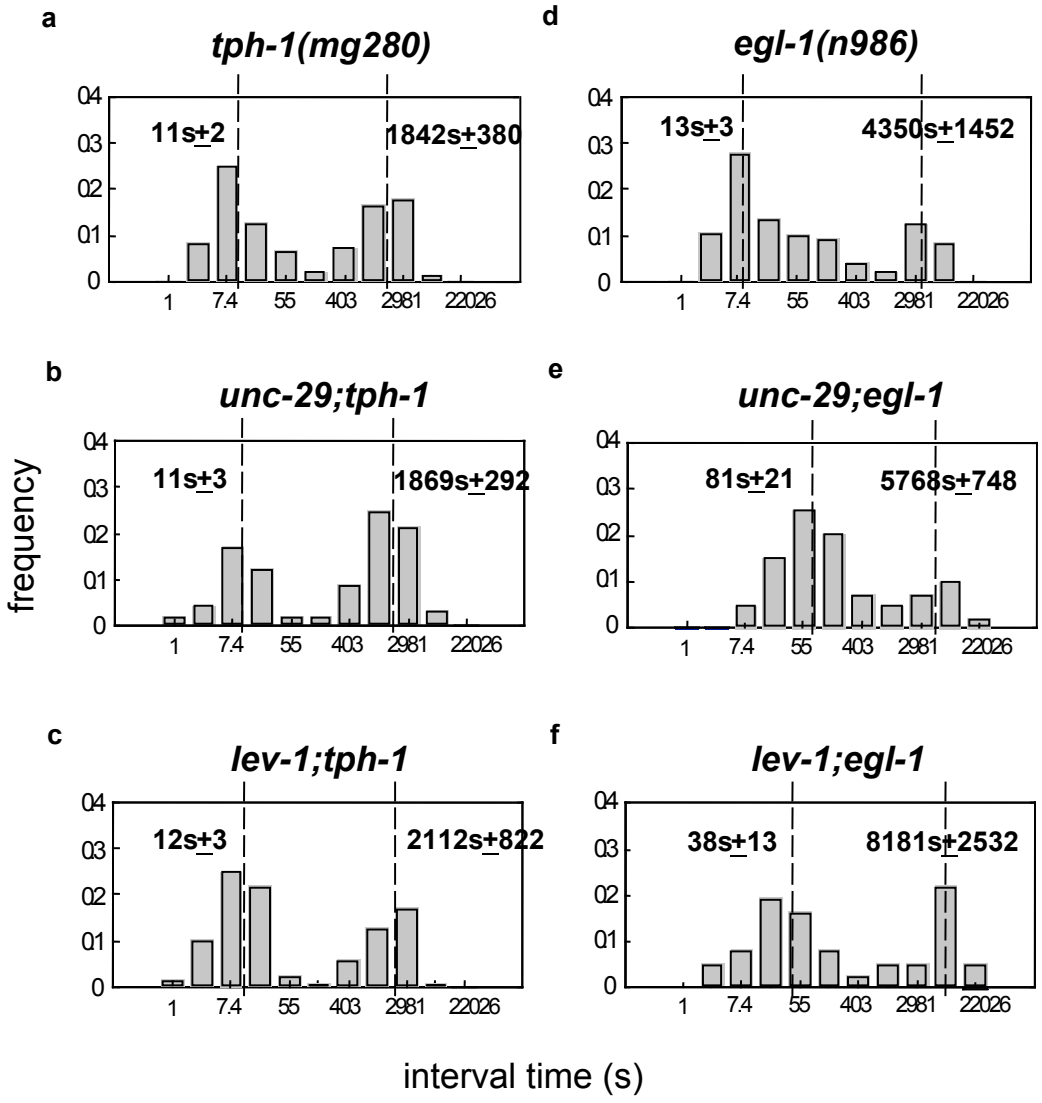



**Figure 5: Interactions between the egg-laying phenotypes of *egl-1, tph-1* and the levamisole receptor genes.** Shown are histograms of intervals times between egg-laying events. Dashed lines indicate the estimated intra-cluster and inter-cluster time constants. The number of animals, hours tracked, and total intervals analyzed were: *tph-1(mg280)*--6 animals, 40 hr, 131 intervals; *tph-1(mg280); lev-1(e211)*--6 animals, 33 hr, 115 intervals; *tph-1(mg280);unc-29(x29)*--6 animals, 33 hr, 88 intervals; *egl-1(n986)*--13 animals, 70 hr, 118 intervals; *egl-1(n986); lev-1(e211)*--4 animals, 48 hr, 35 intervals; *egl-1(n986);unc-29(x29)*--6 animals, 72 hr, 40 intervals. The short intervals (< 300s) in *egl-1; lev-1* and *egl-1; unc-29* double mutants were statistically different from the *egl-1* single mutant according to the Mann-Whitney rank sum test (level of confidence $p < .05$).

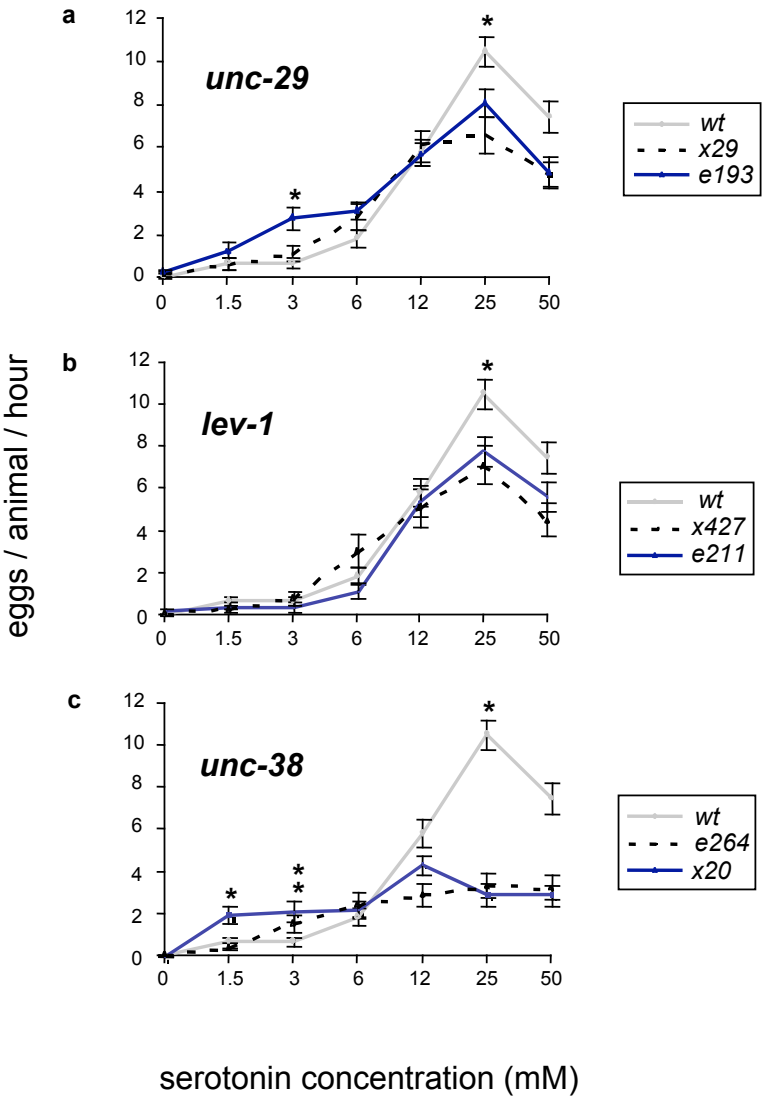



**Figure 6: Effect of levamisole receptor genes on egg-laying in response to serotonin.** Egg-laying responses to serotonin were determined for the different mutant alleles by placing individual animals in liquid M9 and the indicated concentration of serotonin, and counting the number of eggs laid by each animal after 1 hour of drug exposure. Both alleles of *unc-38(x20)* and *unc-38*(*e264)* and one allele of *unc-29(e193)* showed a statistically significant increase in egg-laying at low (3.25 mM) serotonin concentrations according to the Mann-Whitney rank sum test (level of confidence $p < .05$). By the same test, all shown levamisole receptor mutants *unc-29(x29), unc-29(e193), unc-38(x20), unc-38(e264), lev-1(e211),* and *lev-1(x427)* displayed a statistically significant decrease in egg-laying at high (25 mM) serotonin concentrations (level of confidence $p < .05$). Individual points and error bars indicate the mean and SEM of at least the following numbers of trials: N2=70, *unc-29(x29)*=38, *unc-29(e193)*=36, *unc-38(x20)*=36, *unc-38(e264)*=32, *lev-1(e211)*=33, *lev-1(x427)*=34.

## CHAPTER II APPENDIX: GENES INVOLVED IN REGULATION OF THE NICOTINC RECEPTOR

### INTRODUCTION

Long-term exposure to cholinergic agonists such as nicotine causes changes in both the abundance and activity of nicotinic acetylcholine receptors, leading to long-lasting changes in behavior. As the molecular mechanisms responsible for adaptation to nicotine are not well understood in any organism, we have been using the nematode *C. elegans* as a model to further investigate these processes.

Treatment with cholinergic agonists including levamisole and nicotine causes hypercontraction of the body muscle leading to paralysis, increased pharyngeal pumping, and stimulation of egg-laying under conditions which normally inhibit egg-laying. Nematodes adapt to the chronic presence of nicotinic agonists as they regain normal movement patterns and fail to undergo egg-laying stimulation under inhibitory conditions (Waggoner *et al.* 2000). Downregulation of nicotinic receptor abundance is believed to be responsible for these behavioral changes, in particular those related to egg-laying behavior.

In our lab, a screen was conducted to identify genes involved in regulating the abundance and activity of nicotinic receptors. Several mutants with either nicotine hypersensitivity or nicotine-adaptation defective phenotypes were identified. Among these is *nic-1*, a mutant exhibiting strong nicotine hypersensitive and locomotion defective phenotypes. *nic-1* animals are easily scored as they exhibit severely uncoordinated locomotion and a short, fat body. These hypersensitive animals undergo paralysis and are stimulated to lay eggs at doses of nicotine three-fold lower than required for a wild-type response. In addition, *nic-1* animals are hyperactive for egg-laying, showing a pattern similar to that of wild-type animals treated with nicotinic agonists. *nic-1* also exhibits abnormalities in male mating, a behavior known to be controlled by cholinergic motor neurons.

*nic-1* was recently cloned and found to encode a glycosyltransferase with homology to the *S. cervisiae* alg-2p protein, which is involved in n-linked glycoslyation. Previous studies have shown that *nic-1* mutants have reduced levels of the levamisole receptor in vulval muscles and normal levels in body muscles, indicating that the nicotine hypersensitivity of *nic-1* animals is due to a change in receptor activity, not abundance (J. Kim *et al*, unpublished results). These studies are the first to indicate that glycosylation may play a role in the downregulation of body muscle nicotinic receptor activity in *C. elegans*.

**RESULTS**

In order to obtain a greater understanding of the *nic-1* glycosyltransferase and its role in regulation of the nicotinic receptor, we attempted to generate a null allele at the *nic-1* locus using an EMS mutagenesis protocol. EMS is an effective agent for generating G/C - A/T point mutations although it may produce transitions, transversions, deletions and other chromosomal rearrangements.

In this experiment, double mutant animals containing the *nic-1(lj22)X* lesion were mated to flourescent male animals (*him-8(e1489)IV; ljIs1[dpy-20(+) myo2:yc2.1]*) which had been exposed to the mutagen methanesulfonic acid ethyl ester (EMS). Fluorescence was used to score cross progeny as desired cross progeny and double mutant self-progeny are indistinguishable. In order to maximize the frequency of gamete mutation, the males were exposed to the mutagen in the L4/early adult stage as this is the time of greatest germline proliferation. Subsequent mating to the *nic-1(lj22)X; lon-2(e678)X* double mutant produced a few of the desired flourescent, uncoordinated F1 hermaphrodites which were cloned onto individual plates and allowed to self, generating F2 progeny. The resulting F2 generation was screened for uncoordinated worms which were selected and assayed for genotype and nicotine hypersensitivity (Figure 7).

Several EMS mutagenesis screens produced a single mutant suspected of carrying a new mutation in the *nic-1* gene. This new mutant was mapped to the X chromosome and exhibited a more severe nicotine hypersensitivity and uncoordinated phenotype than the *nic-1* mutant, indicating the possibility of a *nic-1* null allele. In addition, the worm was shorter and fatter in morphology and was found to be extremely slow growing with a small brood size.

Although the mutant maps to the X chromosome, sequencing data from the *nic-1* region showed no base pair change, indicating another gene may be carrying the lesion. In this case, the most likely explanation is that the new mutation was isolated by non-genetic, non-complementation and as such, is likely involved in the same pathway with *nic-1*. (Non-genetic, non-complementation occurs when heterozygosity at two loci encoding gene products involved in the same pathway results in insufficient levels of those proteins, leading to a mutant phenotype.)

**DISCUSSION**

The prospect that glycosylation may play a role in the downregulation of nicotinic receptor activity is certainly interesting. Currently, both wild-type, *nic-1* and new mutant animals are being grown and harvested for glycosylation analysis. However, too many unanswered questions concerning this new mutation remain at this time. In the future, we plan to map this mutant with PCR, using Tc1 transposons. Further studies will be needed to determine the effect of both the *nic-1* and new mutant gene products on the processes of glycosylation and ultimately, regulation of nicotinic receptors.

## METHODS

### Strains and Genetic Methods

The chromosomal locations of the genes studied in these experiments are as follows: LGIV: *him-8*; LGX: *nic-1, lon-2*. Routine culturing of *C. elegans* was performed as described (Brenner 1974).

### Mutagenesis Protocol

Flourescent male animals (*him-8(e1489)IV; ljIs1[dpy-20(+) myo2:yc2.1]*) were harvested from NGM plates at the L4/young adult stage with M9 salt solution. Animals were directly transferred to a 50mM methanesulfonic acid ethyl ester (EMS) solution and placed on an oscillating laboratory shaker for four hours. Animals were harvested, rinsed three times with M9 salt solution, and plated on NGM plates for recovery. Healthy, young adult males were selected for mating to the *nic-1(lj22)X; lon-2(e678)X* double mutant. Approximately ten mating plates were used per mutagenesis, each containing ten to twelve males and two or three hermaphrodites. All EMS contaminated waste were neutralized by overnight exposure to 1M NaOH solution.

### Construction of double mutant strains

Double mutants carrying mutations in *nic-1* and *lon-2* loci were constructed by crossing the *lon-2* male single mutants to *nic-1* hermaphrodites and screening the second generation self-progeny for nicotine sensitive worms with a slightly longer and thinner body than the *nic-1* single mutant (phenotypes of *nic-1* single and *nic-1; lon-2* double animals are virtually indistinguishable). The presence of the *lon-2* allele was detected by mating suspected double mutants to *lon-2* males and scoring hermaphrodite cross progeny for the *lon-2* allele.

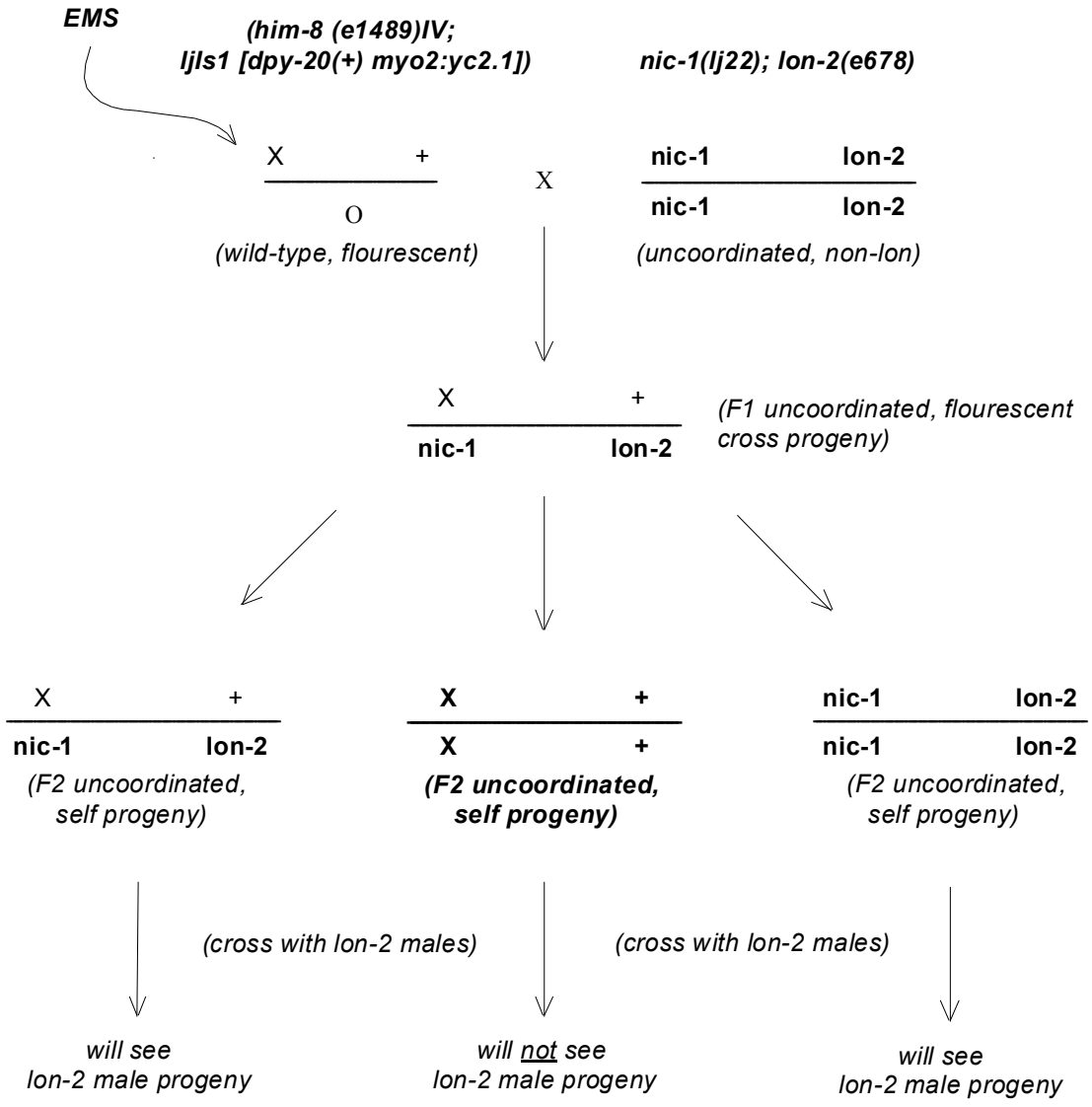



**Figure 7: EMS mutagenesis.** Figure shows EMS mutagenesis scheme. Fluorescent males (*him-8 (e1489)IV;ljIs1 [dpy-20(+) myo2:yc2.1]*) were exposed to the mutagen EMS, generating mutagenized sperm. Flourescent males used to distinguish cross progeny as desired cross progeny and double mutant self-progeny are indistinguishable. Subsequent mating to the *nic-1(lj22); lon-2(e678)* double mutant produced uncoordinated, fluorescent cross progeny which were cloned onto individual plates. Resulting F2 progeny were uncoordinated, thus F2 genotype was assayed by mating to *lon-2(3678)* males to detect the *lon-2* allele. In this cross, the desired F2's will yield no lon male progeny.

# CHAPTER III: AUTOMATED QUANTIFICATIFICATION OF *CAENORHABDITIS ELEGANS* LOCOMOTION BEHAVIOR

## INTRODUCTION

Nicotinic receptors are present in *C. elegans* body muscle where they mediate locomotion. Long-term exposure to cholinergic agonists such as nicotine causes changes in both the abundance and activity of nicotinic acetylcholine receptors, leading to long-lasting changes in behavior, including locomotion. In this study, we have aimed to (i) develop a system for precise quantification of nematode morphology and locomotion behaviors and (ii) use this system to examine the effects of cholinergic agonists on wild-type animals.

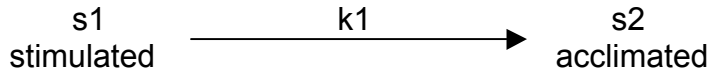
It is possible for an experienced observer to distinguish between several shades of uncoordinated locomotion phenotypes, but this traditional method of detection is highly subjective and prone to error. Many of the terms used by nematode researchers to describe animals with mutant locomotion phenotypes are not only inadequate, but also extremely subjective. In addition, even the most skilled researcher will often be unable to identify mutants that yield subtle phenotypes as well as those genetic defects which manifest themselves over an extended period of time. As such, a great need exists for a reliable, objective, and quantitative behavioral assay for proper evaluation of genes involved in *C. elegans* nervous system function.

## RESULTS

Using an automated tracking and image capture system to record nematode movement and body posture data over a finite period, we have produced a system capable of providing objective, precise quantification of locomotion parameters. As such, this system aims to provide a reliable assay for use in detecting behavioral abnormalities in the nematode *C. elegans*.

The image capture portion of the tracking system consists of a Zeiss Stemi 2000-C dissecting scope fitted with an analog CCD video camera. The video signal from the camera is sent to the video recorder (in this case, a computer hard drive has been utilized, although a video cassette recorder may also be used). The video signal is also sent to the tracking/image boards, which relay this information to the customized PC tracking software. Here, the signal is processed and commands are sent to the stage controller for necessary adjustments to ensure the nematode remains in the field of view (see Figure 8). An 8-bit, grey scale (256 grey levels) image is captured at half second intervals. This image is processed into a binary (black and white) image which is then converted into a thirty-point backbone.

Previous studies have shown that *C. elegans* undergoes behavioral changes in response to light or heavy touch. With this system, we have shown that wild-type nematodes experience an increase in speed following moderate stimulation, which occurs during initial tracking preparation. This hyperactive phase, which we call S1, defers to a state of decreased activity, S2, as the worm becomes acclimated to its new environment and recovers from the stimulation received during experimental preparation. The rate at which this occurs is measured as a single exponential kinetic constant, k1. Below is a simple diagram illustrating this concept.




We have used our tracking instrumentation to examine the effect of nicotine exposure and withdrawal on nematode locomotion behavior in both the stimulated and acclimated states, as well as its effect on the kinetic rate, k1.

Used as controls, naive animals were compared with two pharmacologically treated classes: nicotine treated and nicotine withdrawal nematodes. Both classes were exposed

overnight (16hrs.) to 2mM nicotine media. Following this period, treated animals were tracked for twenty minutes on nicotine plates. Additional data sets were collected from withdrawal animals, which were removed from drug media, and subsequently tracked on plates lacking nicotine at zero, six and twelve hour time points. A total of ten, twenty-minute recordings exist for each class (naive, treatment, and withdrawal). Individual worms were followed throughout the course of the tracking experiment for the withdrawal class.

**Global Movement Speed**

To quantify nematode locomotion behavior, we have used a parameter called global movement speed, which is defined as the distance traveled by the nematode centroid over time. Naïve, wild-type animals experience fluctuations in global speed over the course of a twenty minute recording; initially, they move faster due to stimulation received during experimental preparation, but during tracking, the animals become acclimated to their new environment, exhibiting decreased global speed (Figures 9, 10). This rate of acclimation is measured as a single exponential decay. Thus, in the course of examining global movement speed of wild-type animals, we have quantified and examined three parameters: (i) the stimulated state (S1) speed, (ii) the acclimated state (S2) speed and (iii) the kinetic rate, k1.

To determine the immediate effect of nicotine exposure on *C. elegans* global speed, we have examined treated nematodes, which were placed on nicotine plates overnight and subsequently tracked on nicotine media. Global speed kinetics indicate that chronic agonist exposure induces a moderate increase in acclimated speed. However, treated animals exhibit only slight deviations in their initial (stimulated state) speed and kinetic rate (Figure 9).

The effects of nicotine withdrawal on global movement were examined by tracking nicotine exposed nematodes on normal media at zero, six and twelve hour time points. Withdrawal causes distinct increases in both the acclimated global speed and rate constant, k1.

Although the increase in acclimated global speed subsides at latter time points, the elevation seen in the kinetic rate constant persists following twelve hours of recovery. Concerning speed in the stimulated state, withdrawal animals remain unaffected until the twelve hour time point, at which they exhibit a moderately decreased movement ability (Figure 10).

**Reversal Rate**

In order to extend our quantification of *C. elegans* locomotion behavior, we have examined reversals, which often occur as the nematode is forging or attempting to make a sharp turn. Reversals are detected by first identifying incidences in which the animal moves more than 2.5% of its total length between successive frames. Superimposition of consecutive backbone points is subsequently checked, and any incidence in which the head in the initial frame is not superimposed is considered to be a reversal.

In contrast to global movement, reversal rate does not exhibit a kinetic decay. However, both nicotine treatment and withdrawal lead to significant effects on the reversal rate of nematodes: both treated and withdrawal animals exhibit a nearly three-fold increase in reversal rate as compared with their naïve counterparts (Figure 11).

**Global Movement Scope**

Global movement scope was also used to quantify nematode locomotion behavior. Defined as the greatest distance the worm travels from its initial location, this parameter measures the geographical extent of the animal's locomotion activity.

Analogous to reversal rate, movement scope does not exhibit exponential decay but is affected by nicotine. Specifically, while movement scope of treated animals falls short of naïve levels, withdrawal nematodes deviate significantly in their scope, especially at latter time points (Figure 12).

## DISCUSSION

By using an automated tracking and image capture system to record nematode movement and body posture over an extended time period, we have been able to examine *C. elegans* locomotion behavior in a precise, objective manner.

Using this system to analyze locomotion patterns of naïve, wild-type animals, we have discovered that moderate stimulation is sufficient to induce an initial increase in global speed. Hyperactivity is followed by transition to a state of acclimation, as the worm adjusts to its new environment. This rate of transition is measured by the kinetic constant, k1. In addition, we have also shown that withdrawal from or chronic treatment with nicotine effects not only k1 and global speed in S1 and S2, but reversal rate and global movement scope as well. As such, this instrumentation has allowed us to objectively quantify the process of nicotine tolerance and effects of nicotine withdrawal in *C. elegans* locomotion behavior.

### Global Movement (Speed)

In contrast to our observation that naïve animals become quickly paralyzed when placed on nicotine plates, nematodes treated overnight with nicotine exhibit no reduction in overall locomotion ability and only a slight deviation in the kinetic rate from their naïve counterparts. This contrast suggests that long-term nicotine exposure has little or no effect on overall nematode movement capacity and that *C. elegans* is able to develop a tolerance to chronic agonist exposure (Figure 9). We have also seen that overnight nicotine treatment induces a moderate increase in acclimated global speed, indicating that nicotine acts as a stimulant, at least in the acclimated state.

Withdrawal animals also experience an increase in acclimated speed at all time points, corroborating treated nematode data which suggests that nicotine behaves as a stimulant on *C. elegans* acclimated locomotion speed. Ironically, the kinetic increase seen in withdrawal

animals implies that nicotine acts as a depressant, as it hastens the progression from a stimulated to an acclimated state. This initial kinetic increase does not diminish, but persists at latter time points, indicating an increase in k1 is a long-lasting effect of nicotine withdrawal (Figure 10).

**Reversal Rate**

Although reversal rate does not exhibit a kinetic decay, both nicotine treatment and withdrawal lead to significant effects on the reversal rate of nematodes. Both classes exhibit a nearly three-fold increase in reversal rate from their naïve counterparts (Figure 11), which may be consistent with the increased global speed of these animals. In any event, this increase in reversal rate appears to be a long-lasting effect of nicotine exposure as we see persistence in withdrawal animals at latter time points (Figure 11).

**Global Movement Scope**

Similar to reversal rate, global movement scope is affected by nicotine exposure but does not exhibit a kinetic decay. Specifically, we see a decreased scope and erratic locomotion tracks in both treated and withdrawal animals (Figures 12, 13). While a moderate decline is seen in treated animals, reduced scope appears to be induced by nicotine withdrawal as we observe a significant and progressive decrease in withdrawal nematodes at all time points (Figure 12).

**Tracking and Imaging System**

At this time, we are just beginning to appreciate the potential of this automated behavioral phenotype quantification system. In the future, we plan to utilize this instrumentation to detect strains which are defective in nicotine tolerance and withdrawal, thereby dissecting the pathways responsible for these behavioral processes. We are also eager

to use this system to collect data from nicotinic receptor mutants as well as other strains containing mutations which may be relevant to nematode locomotion.

As previously mentioned, we intend to examine the effects of nicotine treatment on levamisole receptor mutants to further understand the effects of nicotine on *C. elegans* locomotion behaviors. However, we suspect that much of the effects of nicotine on locomotion are mediated through the levamisole-insensitive nicotinic receptor studied in Chapter II. As this receptor is molecularly and genetically uncharacterized, it will be difficult to determine the basis for such response at this time.

We are also interested in investigating the possibility that nicotinic receptors located in the nematode nerve ring (brain) or other interneurons may be responsible for the effects of nicotine withdrawal and chronic exposure on locomotion.

## METHODS

### Strains and Genetic Methods

The chromosomal locations of the genes studied in these experiments are as follows: LGI: *unc-29, unc-38*. The *unc-38* neuronal and muscle partial rescues *unc-38(sy576); him-5(e1490); syEx [punc-119 :: unc-38][pmyo-2 :: gfp], unc-38(sy576); him-5(e1490); syEx [pmyo-3 :: unc-38][pmyo-2 :: gfp]* were also used. Routine culturing of *C. elegans* was performed as described (Brenner 1974).

### Tracking Protocol

The day prior to tracking, approximately thirty L4 animals are scored and transferred to a second plate, where they are allowed to mature into adults. On the day of the tracking experiment, suitable tracking plates were carefully selected from a batch of freshly poured NGM plates, being screened for an even surface and the absence of bubbles or foreign

material. Following selection, plates were seeded with a single drop of an overnight OP50 *E. coli* bacteria culture and allowed to dry for five minutes. Adult animals were randomly picked from the previously scored population onto a seeded tracking plate and were allowed to acclimate for five minutes. Subsequent twenty minute recordings of the worms' movement and body posture were obtained.

For nicotine treatment experiments, wild-type worms were exposed to 2mM nicotine plates for a period of sixteen hours. Randomly selected adults were transferred to and subsequently tracked on nicotine plates at this zero hour time point. Nematodes in these experiments were never removed from 2mM nicotine media.

For nicotine withdrawal experiments, wild-type worms were exposed to 2mM nicotine plates for a period of sixteen hours. Randomly selected adults were selected and tracked on normal media at zero, six and twelve hour time points following removal from nicotine.

**Image Data Collection**

Using a Zeiss Stemi 2000-C dissecting scope fitted with a Cohu High Performance CCD video camera, worm locomotion and body posture were monitored over a defined time period. A tracking apparatus (Parker Automation, SMC-1N) is monitored by custom computer software to focus the worms' position in the center of the field of view. Nematode locomotion and body posture were recorded as grey scale images, which were snapped and saved at 0.5 second intervals for at least twenty minutes. The image was then trimmed to the smallest axis-aligned rectangle that contained the identified nematode, and saved as eight-bit grayscale data. The dimensions of each image and the coordinates of the nematode centroid were also saved simultaneously as the references for the location of an animal in the tracker field at the corresponding time point when the images are captured. The stereomicroscope was fixed to its largest magnification (50X) during operation. Depending on the type and the

posture of a worm, the number of pixels per image frame varied although the number of pixels per millimeter was fixed at 312.5 pixels/mm for all worms.

**Image Data Processing**

Binarization of the grey-scale (256 shades of grey) image is achieved by first measuring the background intensity of a typical tracking plate (NGM media seeded with OP50 *E. coli*). The background was measured at 195 ± 2 for all tracking experiments. Any pixel with an intensity value of 195 ± 5 is designated as background and any pixel with an intensity value falling outside of this threshold is designated as the nematode. The preliminary binary image is first dilated to fill any portions of the worm misassigned as background. This dilated image is then eroded, yielding a final binary image.

The complete binary image is thinned to create a backbone image. Thinning performed as described (Zhang, Suen, 1984).

Subsequently, backbone extraction is achieved from the thinned binary image. The endpoints of the thinned backbone are identified and the backbone itself is divided into twenty-nine equidistant points. This creates a string of thirty consecutive backbone points representing the posture of the nematode.

In order to ensure the head and tail of the worm are correctly identified in each frame, successive backbone images are analyzed in the following manner. Each backbone image is analyzed by drawing a line (designated the "skewer line") which passes through the nematode centroid and is parallel to the angle of the line connecting the head and tail. The initial fame is assigned a value$\tau$. If the next frame, $\tau$+1, is available, and the angle formed by the skewer lines of frames $\tau$ and $\tau$+1 is less than $30^0$, then the head is judged in frame $\tau$+1 as the endpoint nearest the head in frame $\tau$. If a single frame is missing, this angle is dropped to $5^0$ and if the number of missing frames is between two and ten, the angle is reduced to $2^0$. In the event that

ten or more frames are missing or the above criteria are not met, the head must be judged manually.

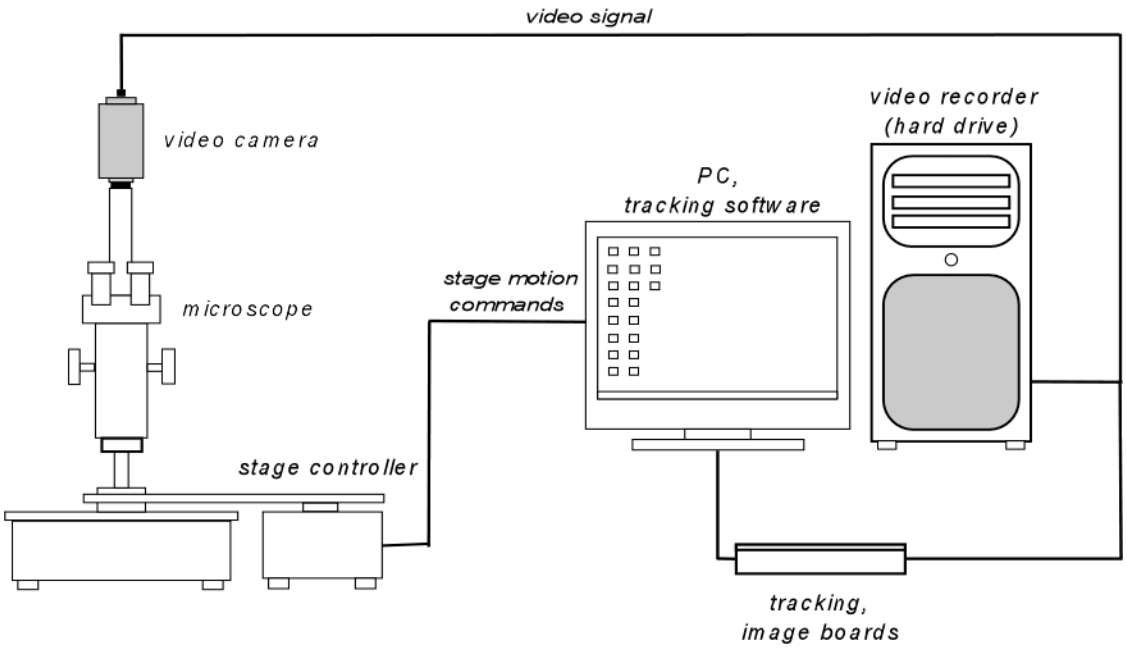



**Figure 8: Tracking system.** A dissecting scope has been fitted with a high-performance, CCD video camera, which captures an image of a single nematode at half-second intervals. The video signal from the camera is sent to both the video recorder (computer hard drive or video cassette recorder) and a pair of tracking and image boards. Customized tracking software evaluates the signal from tracking/image boards and sends commands to the stage controller for necessary adjustments to ensure the nematode remains in the center of the field of view.

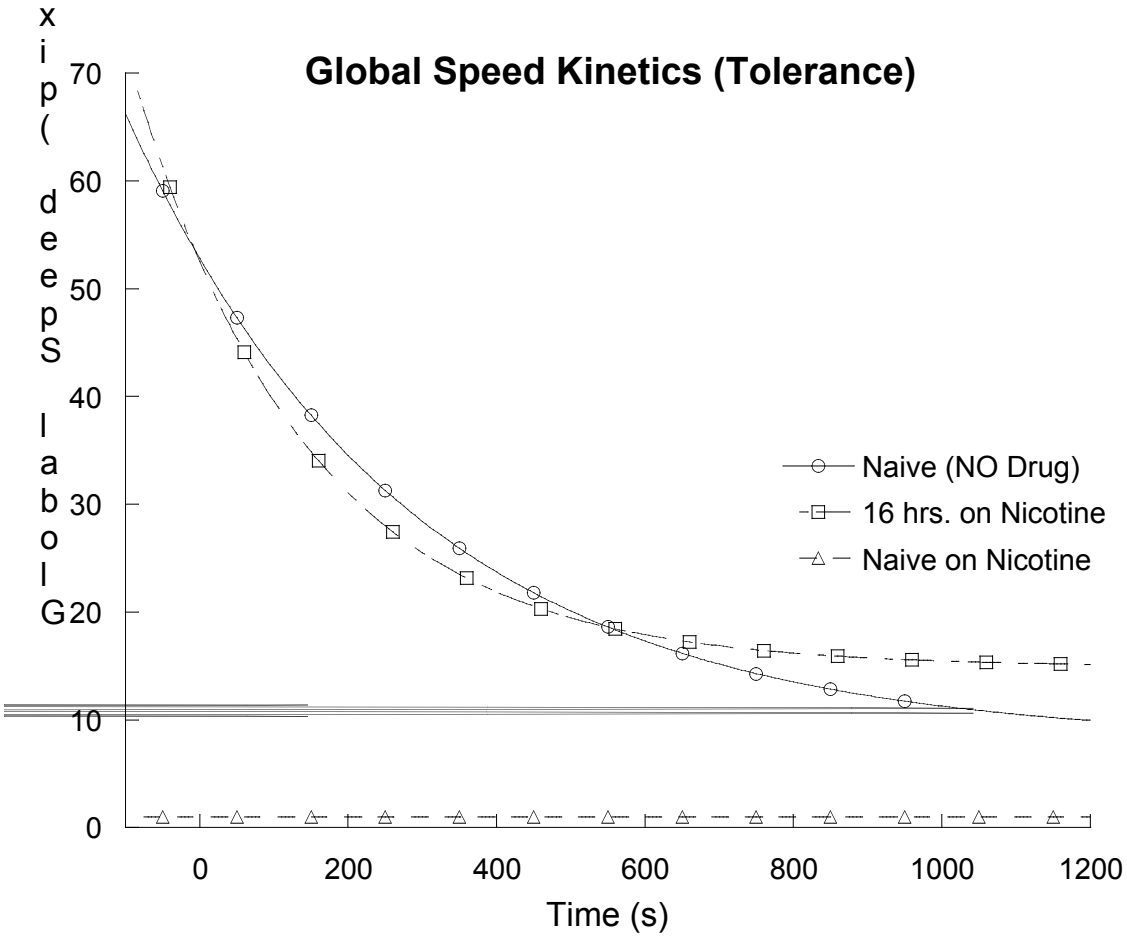



**Figure 9: Global speed kinetics (Tolerance).** Global speed is defined as the distance traveled by the worm centroid vs. time. Shown above are the global speed kinetics of naïve (no drug), nicotine treated animals (16hrs. on nicotine) and naïve animals on nicotine media (observed). Chronic nicotine exposure confers a moderate increase in acclimated speed, suggesting nicotine behaves as a stimulant in the acclimated state. In contrast to our observation that naïve animals experience nearly immediate, long-lasting paralysis when placed on nicotine plates, animals sustaining chronic (16hr.) exposure to nicotine exhibit only slight deviations in initial (stimulated state) locomotion ability and kinetic rate from their naïve counterparts, indicating that the nematode is able to tolerate and adapt to chronic agonist exposure.

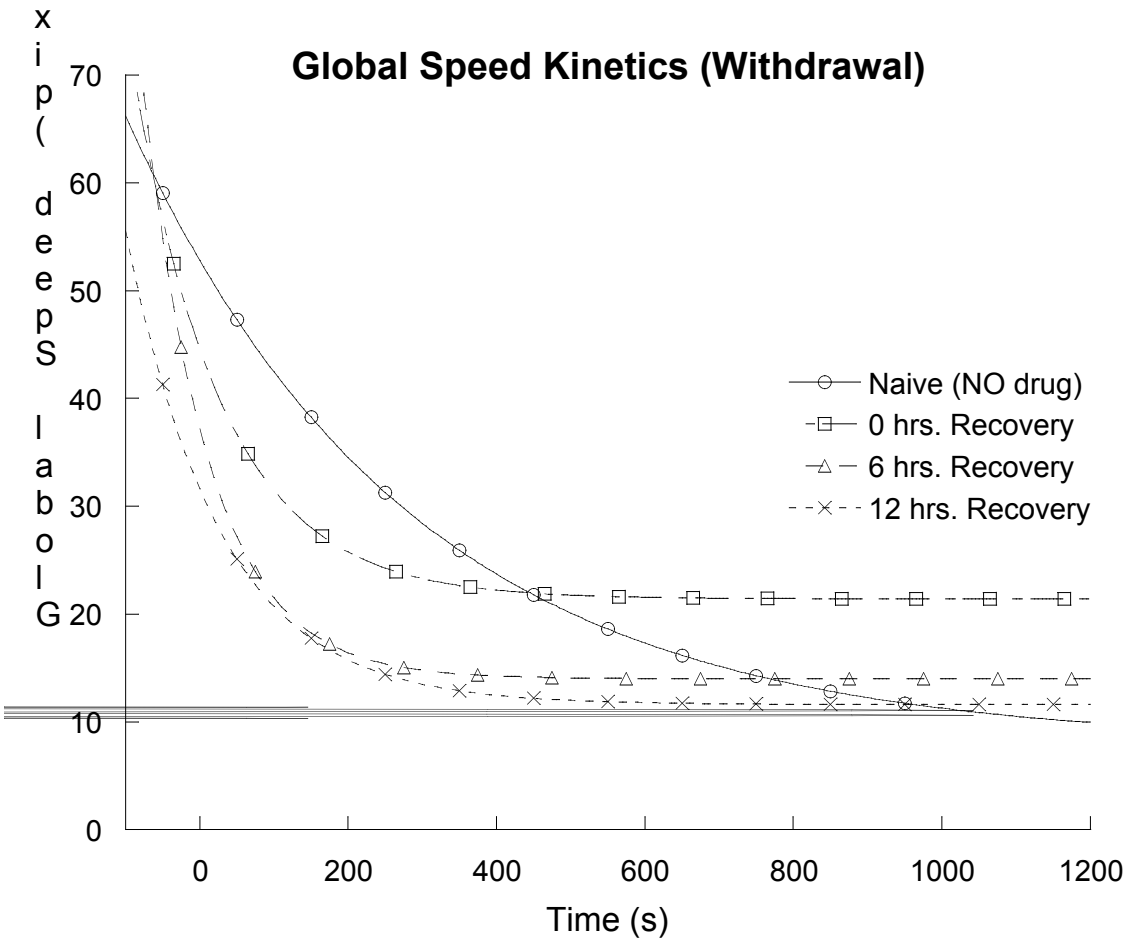



**Figure 10: Global speed kinetics (Withdrawal).** Global speed is defined as the distance traveled by the worm centroid vs. time. Shown above are the global speed kinetics of nicotine withdrawal animals. Nematode speed in the stimulated state appears to be initially unaffected, becoming altered only after twelve hours of removal. Withdrawal leads to an increase in acclimated state speed, though this hyperactivity declines with time, suggesting nicotine acts as a stimulant. The kinetic decay value, k1, also experiences an initial increase in withdrawal animals, which persists at latter time points, indicating an elevated kinetic constant is an effect of nicotine withdrawal.

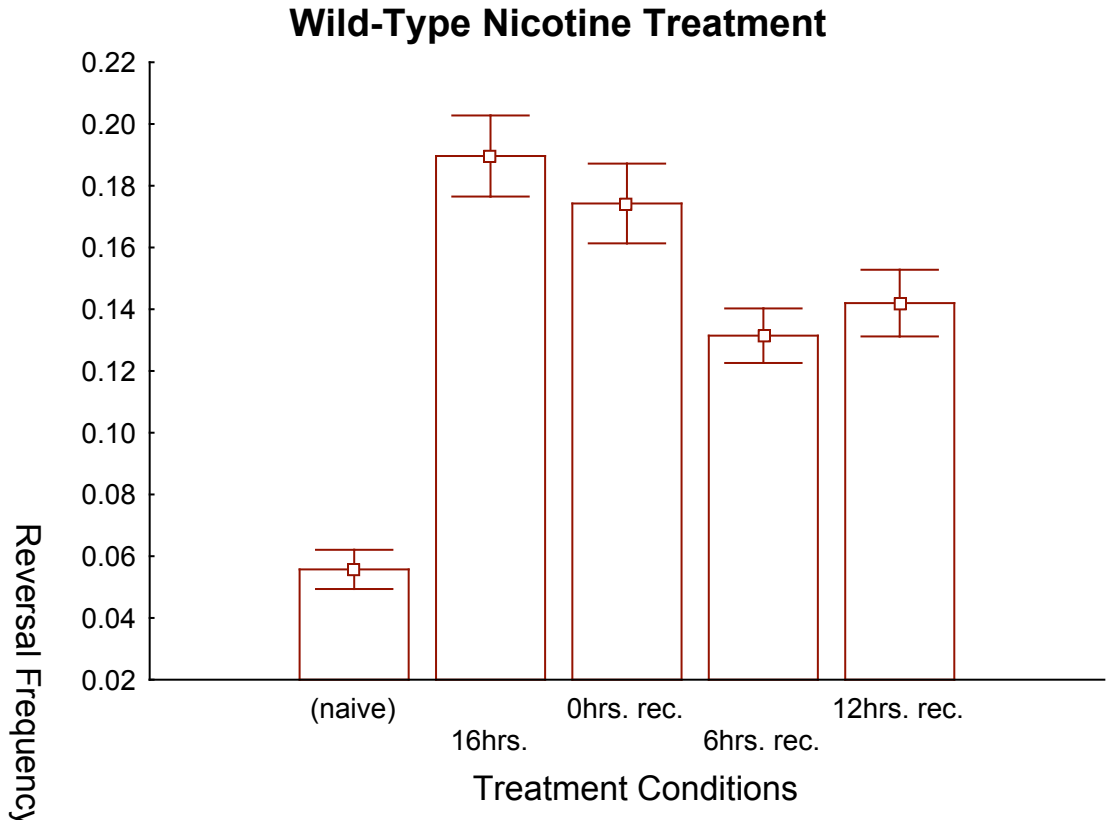



**Figure 11: Reversal frequency.** This graph displays the reversal frequencies for naïve and nicotine treated nematodes. Reversal events are quantified by first detecting global movement at a rate greater than 2.5% of the worms' total length. Subsequently, superimposition of consecutive backbone points is checked. Any incidence in which the head in the initial frame is superimposed and the tail is not, is considered a reversal. Data indicates that both classes exhibit an elevated reversal rate, which appears to be a long-lasting effect of nicotine exposure as we see persistence in withdrawal animals at latter time points.

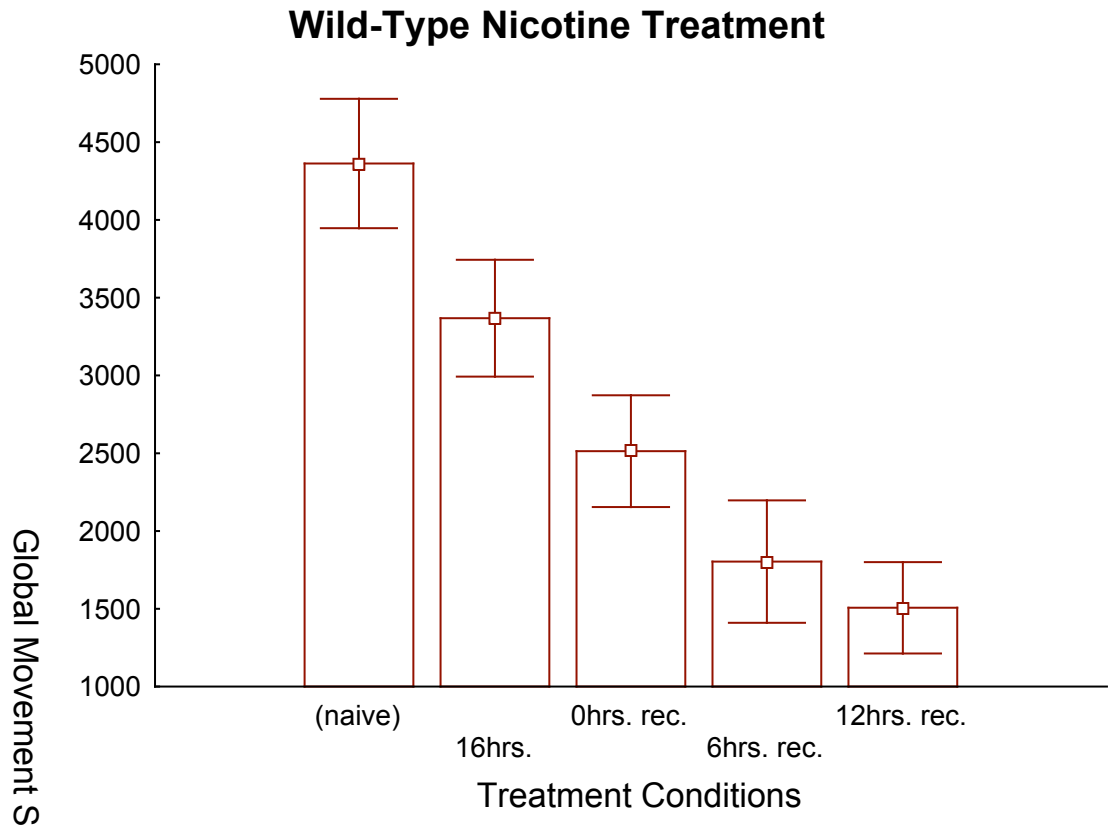



**Figure 12: Global movement scope.** This graph displays the global movement scope of naïve and nicotine treated worms. Global movement scope is defined as the greatest distance the worm travels from its initial location, measuring the geographical extent of locomotion activity. Nicotine exposure leads to a significant decrease in movement scope, which appears to be an effect of nicotine withdrawal as illustrated by the significant and progressive decrease in movement scope exhibited by withdrawal animals at the six and twelve hour time points.

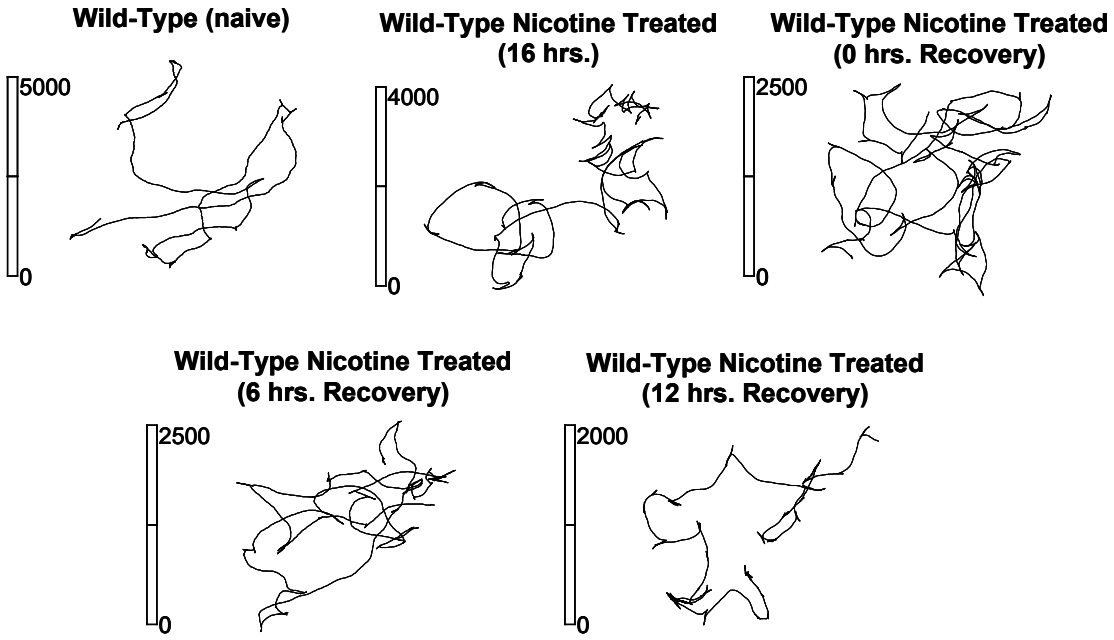



**Figure 13: Locomotion Tracks.** The images above show the movement of naïve and nicotine treated nematodes as a single point (centroid) relative to their initial tracking position, illustrating the effect of nicotine treatment and withdrawal on wild-type locomotion. Note the erratic movement, decrease in scope, and increase in reversal frequency of treated and withdrawal nematodes.

## CHAPTER V: CONCLUSION

In this thesis, I have examined the role of nicotinic acetylcholine receptors in *Caenorhabditis elegans* egg-laying and locomotion behavior. I have shown evidence for the existence of a second, genetically uncharacterized nicotinic receptor functioning in *C. elegans* egg-laying behavior (Figure 2). In addition, I have participated in the development and implementation of an automated behavioral phenotype quantification system, which is capable of reliable and objective evaluation of nematode locomotion parameters. This system was used to examine the effects of nicotine treatment and withdrawal on *C. elegans* locomotion behavior.

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